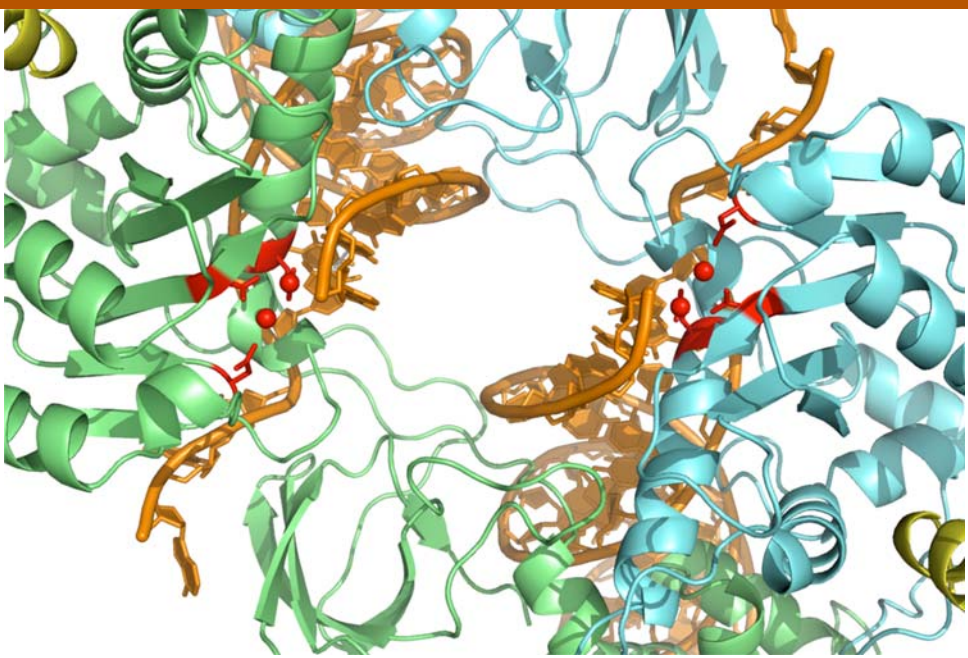
Abstracts of papers presented
at the 2010 meeting on

RETROVIRUSES

May 24–May 29, 2010



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

Abstracts of papers presented
at the 2010 meeting on

RETROVIRUSES

May 24–May 29, 2010

Arranged by

Christopher Aiken, *Vanderbilt University*
Jeremy Luban, *University of Geneva, Switzerland*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Front cover: Armed and Dangerous. IN tetramer (green, blue and yellow) assembled on the viral DNA ends (orange). The active sites are engaged with 3'-ends of the viral DNA, lying in wait for host DNA to carry out integration. The IN active site residues are painted red and shown as sticks; red spheres are catalytic metal cations. (see Abstract #336 for more details); courtesy of Stephen Hare and Peter Cherepanov, Imperial College London.

Back cover: Group photo from the 1955 Summer Student Research Program at the Jackson Laboratories. Photo courtesy of the Jackson Laboratory Archives.

RETROVIRUSES

Monday, May 24 – Saturday, May 29, 2010

Monday	7:30 pm	**1** Entry
Tuesday	9:00 am	**2** RT and TRIM5
Tuesday	2:00 pm	**3** Goin' Nuclear
Tuesday	7:30 pm	Keynote Speaker I
Tuesday	8:30 pm	**4** Poster Session I
Wednesday	9:00 am	**5** APOBEC
Wednesday	2:00 pm	**6** Poster Session II
Wednesday	4:30 pm	Wine and Cheese Party*
Wednesday	7:30 pm	**7** Integration
Thursday	9:00 am	**8** RNA
Thursday	2:00 pm	**9** Assembly
Thursday	7:30 pm	Keynote Speaker II
Thursday	8:30 pm	**10** Poster Session III
Friday	9:00 am	**11** Tetherin and XMRV
Friday	2:00 pm	**12** Structure, Genome and Defense
Friday	4:00 pm	Special Celebration: Keynotes III and IV
Friday	6:00 pm	Banquet
Saturday	9:00 am	**13** Tetherin and Pathogenesis

Poster sessions are located in *Bush Lecture Hall*

* *Airslie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:

Breakfast 7:30 am-9:00 am

Lunch 11:30 am-1:30 pm

Dinner 5:30 pm-7:00 pm

Bar is open from 5:00 pm until late

In Memoriam

With great sadness we report the death of Dr. Daniel Wolf, an extraordinary and productive scientist, of an aggressive chondrosarcoma. Dan was working in Steve Goff's lab at the time of his death last fall at age 32. Dan was responsible for uncovering many aspects of the silencing of retroviral DNAs in embryonic stem cells, and presented often at this meeting. His beautiful science, enthusiasm, and generosity will be sorely missed.

PROGRAM

MONDAY, May 24—7:30 PM

SESSION 1 ENTRY

Chairpersons: **J. Mak,** The Burnet Institute, Melbourne, Australia
C. Berlioz-Torrent, INSERM U1016, Paris, France

SESSION 2 RT AND TRIM5

Chairpersons: **F. Diaz-Griffero,** Albert Einstein College of Medicine, Bronx, New York
W. Johnson, Harvard Medical School, Boston, Massachusetts

SESSION 3 GOIN' NUCLEAR

Chairpersons: **M. Naghavi,** University College Dublin, Ireland
E. Bacharach, Tel Aviv University, Israel

TUESDAY, May 25—7:30 PM

KEYNOTE SPEAKER

Sandra Ruscetti
National Cancer Institute-Frederick

"Understanding the biological effects of the human retrovirus XMRV—Lessons learned from studying murine retroviruses"

TUESDAY, May 25—8:30 PM

SESSION 4 POSTER SESSION I

SESSION 5 APOBEC

Chairpersons: **V. Simon,** Mount Sinai School of Medicine, New York, New York
Y.-H. Zheng, Michigan State University, East Lansing

SESSION 6 POSTER SESSION II

A novel host factor, ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1), influences HIV-1 infection in human monocytic cells
Nidhanapati K. Raghavendra, Heather L. Hoy, Li Wu.
Presenter affiliation: Ohio State University, Columbus, Ohio. 203

Barriers to intergroup HIV-1 recombination revealed by a new dual-marker system
Olga A. Nikolaitchik, Andrea Galli, Michael D. Moore, Vinay K. Pathak, Wei-Shau Hu.
Presenter affiliation: National Cancer Institute, Frederick, Maryland. 204

Reconstruction of HERV-F(c)1, associated with multiple sclerosis
Kari K. Nissen, Finn S. Pedersen, Bjørn A. Nexo.
Presenter affiliation: University of Aarhus, Aarhus C, Denmark. 205

WEDNESDAY, May 26—4:30 PM

Wine and Cheese Party

WEDNESDAY, May 26—7:30 PM

SESSION 7 INTEGRATION

Chairpersons: **M. Roth,** UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey
P. Cherepanov, Imperial College London, United Kingdom

Lens epithelium derived growth factor (LEDGF) fusion proteins that redirect HIV-1 DNA integration
Andrea L. Ferris, Xiaolin Wu, Christina M. Hughes, Claudia Stewart, Steven J. Smith, Thomas A. Milne, Gang G. Wang, Ming-Chieh Shun, C. David Allis, Alan Engelman, Stephen H. Hughes.
Presenter affiliation: National Cancer Institute-Frederick, Frederick, Maryland. 206

Recombinant Ty3 integrase mediates Brf1c-dependent and sequence-specific strand transfer *in vitro*
Xiaojie Qi, Kim Nguyen, Heather Hatzis, Suzanne Sandmeyer.
Presenter affiliation: University of California Irvine, Irvine, California. 207

THURSDAY, May 27—9:00 AM

SESSION 8 RNA

Chairpersons: **J.-C. Paillart,** CNRS-Université de Strasbourg, France
W.-S. Hu, National Cancer Institute-Frederick, Maryland

THURSDAY, May 27—2:00 PM

SESSION 9 ASSEMBLY

Chairpersons: **F. Bouamr,** NIAID, National Institutes of Health, Bethesda, Maryland
A. Ono, University of Michigan Medical School, Ann Arbor

Rab11a modulates HIV-1 Env incorporation
MIngli Qi, Jaang-Jiun Wang, Hin Chu, Lingmei Ding, Paul Spearman.
Presenter affiliation: Children's Healthcare of Atlanta and Emory University School of Medicine, atlanta, Georgia. 241

THURSDAY, May 27—7:30 PM

KEYNOTE SPEAKER

Ruslan Medzhitov
Howard Hughes Medical Institute
Yale University

THURSDAY, May 27—8:30 PM

SESSION 10 POSTER SESSION III

Lipid depletion modulates TRIM5a -mediated retroviral restriction
Seiga Ohmine, Ryuta Sakuma, Yasuhiro Ikeda.
Presenter affiliation: Mayo Clinic, Rochester, Minnesota. 242

The localization of APOBEC3H haplotypes in HIV-1 virions determines its antiviral activity
Marcel Ooms, Susan Majdak, Christopher Seibert, Ariana Harari, Viviana Simon.
Presenter affiliation: Mount Sinai School of Medicine, New York, New York. 243

Gag RNA and membrane binding determinants function coordinately during HIV-1 Gag particle assembly
David Ott, Lori Coren, Teresa Shatzer.
Presenter affiliation: SAIC-Frederick, Inc, NCI-Frederick, Frederick, Maryland. 244

Adaptation of HIV-1 to escape rhesus monkey TRIM5-a
Beatriz Pacheco, Andres Finzi, Joseph Sodroski.
Presenter affiliation: Dana-Farber Cancer Institute, Boston, Massachusetts; Harvard Medical School, Boston, Massachusetts. 245

FRIDAY, May 28—9:00 AM

SESSION 11 TETHERIN AND XMRV

Chairpersons: **S. Neil,** King's College London, United Kingdom
I. Singh, University of Utah, Salt Lake City

Investigations into XMLV-related virus infection
Harriet C. Groom, Virginie C. Boucherit, Kate N. Bishop.
Presenter affiliation: MRC National Institute for Medical Research, London, United Kingdom. 334

FRIDAY, May 28—2:00 PM

SESSION 12 STRUCTURE, GENOME AND DEFENSE

Chairpersons: **F. Bushman,** University of Pennsylvania, Philadelphia
D. Unutmaz, New York University School of Medicine, New York

A layered structure in the HIV-1 gp120 inner domain that regulates gp41 interaction and the transition into the CD4-bound conformation is conserved among SIV envelope glycoproteins
Andrés Finzi, Shi-Hua Xiang, Marie Pancera, Beatriz Pacheco, Liping Wang, Peter D. Kwong, Joseph Sodroski.
Presenter affiliation: Dana Farber Cancer Institute, Boston, Massachusetts. 335

Structural bases of retroviral intasome assembly, activity and inhibition
Stephen Hare, Saumya Shree Gupta, Alan Engelman, Peter Cherepanov.
Presenter affiliation: Imperial College London, London, United Kingdom. 336

Lentiviral vector mediated gene therapy for beta-thalassemia: Transfusion independence and activation of HMGA2
Troy Brady, .
Presenter affiliation: University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania. 337

Diverse families of non-retroviral viruses identified as endogenous elements in avian and mammalian genomes
Aris Katzourakis, Robert J. Gifford.
Presenter affiliation: Rockefeller University, New York, New York. 338

Capsid-specific retroviral restriction factor TRIM5 is a multifunctional E3 ubiquitin ligase that promotes innate immune signaling
Thomas Pertel, Stephane Hausmann, Josefina Lascano, Christian Reinhard, Jessica Guerra, Damien Morger, Sara Züger, Pradeep Uchil, Walther Mothes, Markus Grütter, Jeremy Luban.
Presenter affiliation: University of Geneva, Geneva, Switzerland. 339

A cryptic sensor for HIV-1 activates innate immunity in dendritic cells
Nicolas Manel, Brandon Hogstad, Yaming Wang, David E. Levy, Dan R. Littman.
Presenter affiliation: New York University Medical Center, New York, New York; CNRS-UMR5535, Montpellier, France. 340

FRIDAY, May 28—4:00 PM

SPECIAL CELEBRATION

35th anniversary of the Nobel Prize in Physiology or Medicine, "for their discoveries conerning the interaction between tumor viruses and the genetic material of the cell"

Introduction by John Coffin

Rayla Temin
University of Wisconsin-Madison

Introduction by Stephen Goff

David Baltimore
California Institute of Technology

FRIDAY, May 28

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

SESSION 13 TETHERIN AND PATHOGENESIS

Chairpersons: **D. Evans,** Harvard Medical School, Boston, Massachusetts
M. Pizzato, University of Geneva, Switzerland

XMRV TROPISM IN HEMATOPOIETIC CELLS

Stig M Jensen[1], KyeongEun Lee[2], Cari Petrow-Sadowski[3], Ying Huang[1], Daniel C Bertolette[1], Vineet N KewalRamani[2], Kathryn S Jones[3], Francis W Ruscetti[1]

[1]National Cancer Institute, Laboratory of Experimental Immunology, Frederick, MD, 21702-1201, [2]National Cancer Institute, HIV Drug Resistance Program, Frederick, MD, 21702-1201, [3]Basic Science Program, SAIC-Frederick, Laboratory of Experimental Immunology, Frederick, MD, 21702-1201

Gammaretroviruses have been studied for more than thirty years, yet until recently no strains had been recognized in man. Virus chip screening assays revealed the presence of xenotropic murine leukemia virus-related virus (XMRV) in human tissue, and subsequent reports have suggested XMRV association with both prostate cancer and chronic fatigue syndrome (CFS). Antibodies directed against XMRV have been identified in serum from CFS patients, and replicating virus has been isolated from both serum and activated peripheral blood mononuclear cells (PBMCs). Several studies have indicated that the virus is difficult to detect in the PBMCs of infected individuals. Following activation of *ex vivo* cultured PBMCs, expression of XMRV is increased due to spread of the virus in the culture, which can be blocked by the addition of AZT. Although XMRV has been detected in PBMCs, the published studies on tropism of XMRV have not focused on hematopoietic cells. As one approach to address this, two different forms of soluble XMRV SU proteins were generated and the level of XMRV SU binding to various hematopoietic cell lines and primary cells was determined. The SU proteins bound at detectable levels to several of the hematopoietic cell lines and primary hematopoietic cells, but not to the non-susceptible murine cell line NIH3T3. The ability of XMRV to infect different freshly isolated hematopoietic cells is currently being tested. The ability of XMRV to infect cells at a variety of states of activation and differentiation conditions are also being assessed.

IDENTIFICATION OF THE FIRST CELLS INFECTED DURING VAGINAL TRANSMISSION IN THE DEPO-PROVERA RHESUS MACAQUE MODEL

Katharina Rothwangl[1], Ronald S Veazey[2], Thomas J Hope[1]

[1]Northwestern University, Cell and Molecular Biology, Chicago, IL, 60611,
[2]Tulane University, Veterinary Pathology, Covington, LA, 70433

There is currently a significant effort to develop interventions to prevent the transmission of HIV. To facilitate these efforts, a better understanding of how the virus can breach mucosal epithelial barriers is required. We have previously explored the interaction of HIV with the female genital tract using human explants. This analysis revealed that HIV could penetrate both the columnar epithelium of the endocervix and the squamous epithelium of the vagina and ectocervix. Establishing whether the penetrating virus could lead to productive infection however remained to be elucidated. To identify the first infected cells, we utilized a SIV based vector system that would express the mCherry fluorescent protein. The vector was pseudotyped with the R5 tropic HIV envelope JRFL and concentrated by ultracentrifugation. Depo-Provera treated female macaques were vaginally exposed to the inoculum. Four days later the animals were sacrificed, their genital tracts excised, and cryosections of the tissue were analyzed by fluorescent microscopy. Analysis revealed large but rare foci of red fluorescent cells. The expression of mCherry within these cells was confirmed by emission spectrum analysis. These rare foci of transduced cells were found in areas protected by both the columnar and squamous epithelium. The target cells are primarily CD4+ T cells that have infiltrated into the squamous epithelium or reside just below the columnar epithelium. The clustering of transduced cells suggests that there are small regions within the female genital tract that are susceptible to infection. Understanding the mechanism that leads to this increased local susceptibility could lead to novel treatments that can reduce the male-to-female acquisition of HIV.

EVOLUTION OF ENTRY PHENOTYPE AND HIV PATHOGENESIS

William Ince[1], Gretja Schnell[2], Ron Swanstrom[3]

[1]UNC Chapel Hill, Curriculum in Genetics and Molecular Biology, Chapel Hill, NC, 27599-7295, [2]UNC Chapel Hill, Department of Microbiology and Immunology, Chapel Hill, NC, 27599-7295, [3]UNC Chapel Hill, UNC Center For AIDS Research, Chapel Hill, NC, 27599-7295

The HIV-1 surface Env protein mediates entry into the host cell. Evolution of the *env* gene is a hallmark of infection, and in several ways this evolution has been linked to viral pathogenesis within the context of the disease course. To the extent this evolution affects protein function, the link to pathogenesis may be reflected in the entry phenotype. We have previously observed that HIV-1 grown in humanized mice evolves an Env protein that assumes a more open conformation, enhancing neutralization by an anti-V3 antibody by 1000-fold. This open conformation leads to tighter binding to CD4 and the ability to use low levels of CD4 to enter cells, a hallmark of macrophage tropism. In addition, the open conformation potentiates a low level of CXCR4 tropism. In the humanized mouse model this open conformation preceded the evolution of an X4 virus. Two major determinants of this open conformation are within a length-invariant region between the length-variable domains of V1 and V2. We have also isolated viral *env* genes from the CSF of subjects with HIV-associated dementia, and in some subjects the encoded Env proteins found in the CSF (but not in the blood) are able to mediate entry at low CD4 density, consistent with macrophage tropism. Genetic markers for this activity appear to be located, at least in part, in this same region between V1 and V2. We propose that one type of immunodeficiency may be the loss of anti-V3 antibodies which allows the virus to evolve an Env protein with a more open conformation allowing tighter CD4 binding, using this V1/V2 spacer region as a conformational switch. This potentiates a latent CXCR4 tropism that may allow evolution of X4 viruses, and the ability to infect new cell types with low levels of CD4, such as macrophages and microglia as one path to HIV-associated encephalitis. An initial screening of plasma samples has identified several examples of infected subjects who do not have enhanced neutralization of virus pseudotyped with an Env protein with an open conformation, suggesting the absence of anti-V3 antibodies. We predict that these subjects are likely to have Env proteins with an open conformation and that these subjects may be at risk for more rapid disease progression.

VARIABLE REGIONS OF HIV ENVELOPE CONSTITUTE BARRIERS TO GENETIC CROSSING

Meriem S Hamoudi[1], Etienne Simon-Loriere[1], Paola Rossolillo[1], Andres Finzi[2], Joseph Sodroski[2], Matteo Negroni[1]

[1]Institut de Biologie Moleculaire et Cellulaire, CNRS - Life Science, Strasbourg, 67084, France, [2]Dana-Farber Cancer Institute , Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute , Boston, MA, 02115

Recombination is a frequent source of genetic variability in retroviruses. In HIV it has been shown to be a central evolutionary mechanism contributing to adaptation to environmental constraints. The remarkable genetic diversity of HIV provides a phenomenal tool for defining barriers to genetic crossing between viral strains. These barriers can lead to the identification of vulnerable aspects of HIV biology and on the identification of potential targets for antiviral strategies.
We recently characterised the steps leading from generation to selection of intersubtype recombinant forms generated between primary isolates in the envelope gene (Simon-Loriere et al, PLoS Path. 2009). We observed that the combined effect of recombination mechanism and of purifying selection acting against dysfunctional envelopes explains most of the pattern of recombinant forms found for this gene in the database. In particular, a region poorly tolerant to recombination could be identified in the C2 region of gp120 gene of most isolates studied.
We now identify the source of this fragility in the rearrangement of the variable loops that surround C2. Indeed, the V1/V2 region and, to a lesser extent, the V3 loop, constitute important barriers to genetic crossing between primary isolates belonging to different subtypes of HIV-1 M group. The great variability of these regions is therefore not only limited by their implication in essential mechanisms for viral infectivity, as the formation of the coreceptor binding site, but also is constrained by a strong network of epistatic interactions with other portions of the protein. Evolution of these loops seems therefore to be paralleled by coevolution of other yet-to-define portions. Their identification will help in defining the tridimensional arrangement of the envelope proteins, particularly of gp120, also in the context of the functional trimer. This issue, still debated, is central for the potential development of new antiviral strategies targeting this gene.

INSIDE-OUT CONTROL OF HIV-1 PARTICLE FUSION VIA ALLOSTERIC CHANGES IN ENV CONFORMATION

Amanda S Solis, Christopher Aiken

Vanderbilt University, Microbiology and Immunology, Nashville, TN, 37232

To become infectious, HIV-1 particles undergo a maturation process involving proteolytic cleavage of the Gag polyprotein into its components MA, CA, and NC. Mature HIV-1 virions contain a metastable conical core composed of CA that is competent for uncoating. By contrast, immature particles contain a highly stable spherical Gag lattice. Our laboratory previously reported that immature HIV-1 particles retain the Env glycoprotein complex gp120/gp41 even after extensive lipid removal by treatment with non-ionic detergent (Wyma et al., J. Virol. 74:9381-87, 2000). This observation suggested that the tight association of the long gp41 cytoplasmic tail (CT) with the immature viral core might regulate HIV-1 fusion. Indeed, we and others later showed that immature HIV-1 particles are inhibited for fusion with susceptible target cells (Murakami and Freed, J. Virol. 78:1026-31, 2004; Wyma et al., J. Virol. 78:3429-3435, 2004). The fusion impairment was relieved by truncation of the gp41 CT. The molecular mechanism by which HIV-1 fusion is coupled to cleavage of Gag is not known. We hypothesized that processing of Gag alters the conformation of Env on the viral surface in a gp41 CT-dependent manner. To test this, we quantified the binding of specific monoclonal antibodies to mature and immature HIV-1 particles by immunofluorescence imaging. The results revealed differences in specific epitopes on the surface of mature and immature HIV-1 particles. In particular, the membrane proximal external region (MPER) epitope of gp41 undergoes a conformational change upon maturation resulting in decreased exposure. The differences in epitope exposure in mature and immature particles were abolished by truncation of the gp41 CT, thus linking the fusion defect with Env conformation. We conclude that conformational differences in Env on the surface of mature and immature HIV-1 particles may contribute to the repression of immature particle fusion. Coupling HIV-1 fusion competence to maturation is likely to enhance the overall efficiency of cell-to-cell HIV-1 transmission by preventing premature entry, thus promoting HIV-1 dissemination and persistence in vivo. Our results also have practical implications for vaccine approaches targeting MPER epitopes.

LINKAGE BETWEEN HIV-1 PHYSICAL PROPERTIES AND ENTRY ACTIVITY.

Nitzan Kol*[1], Hongbo Pang*[2], Michael S Kay[2], Itay Rousso[1]

[1]Weizmann Institute of Science, Dept. of Structural Biology, Hertzel Street, Rehovot, 76100, Israel, [2]University of Utah School of Medicine, Dept. of Biochemistry, 15 N Medical Drive East RM, Salt Lake city, UT, 84112

By performing indentation assays on HIV-1 we found that the immature state is 14 fold stiffer than the mature state. Truncation of the cytoplasmic domain of gp160 (ΔCT-gp160) leads to a dramatic decrease in the stiffness of immature viruses, bringing it close to that of mature HIV particles that can enter cells with high efficacy. This is in line with the observation that truncation of the cytoplasmic domain is known to significantly increase the ability of immature HIV to enter cells. Based on this result we proposed that the markedly enhanced stiffness of the shell of immature HIV virus precludes structural deformations necessary for efficient fusion with the cell membrane. Here we show that the stiffness of the immature virion can be restored by incorporating gp160 tail fused to a trans-membrane domain and GFP (denoted as TM1). Moreover, the particle stiffness gradually increases as the amount of TM1 incorporation is elevated. To demonstrate the inverse correlation between stiffness and cell entry further, we prepared a series of immature HIV samples having a constant amount of ΔCT-gp160 and variable amounts of TM1. We find that as TM1 incorporation increases, entry activity is significantly reduced. Intriguingly, the stiffness of the particles is proportionally inversed with entry activity. To rule out the possibility that entry activity is governed by interactions between the intra- and extra-cellular segments of gp160, TM1 and ΔCT-gp160 respectively, the latter was replaced with VSVg envelope protein. Similar entry activity reduction is observed when TM1 is incorporated into immature particles having VSVg on their surface. As a control, we show that TM1 incorporation has little impact on mature entry activity. Based on our findings we conclude that the virus physical properties are mechanistically linked with entry.

*authors contributed equally

MECHANISM OF MLV CELL-TO-CELL TRANSMISSION

Jing Jin, Xaver Sewald, Walther Mothes

Yale University, Microbial Pathogenesis, New Haven, CT, 06510

The ability of retroviruses to efficiently spread from cell to cell contributes to the pathogenesis of virally induced diseases. We have studied the murine leukemia virus (MLV) as a model system to understand the mechanism by which viruses spread from cell to cell. Co-cultures of MLV infected cells with uninfected target cells have revealed that the interaction between the viral glycoprotein (Env) expressed on the infected cell and viral receptor, expressed on the target cells, establishes cell-cell adhesion. If cell-cell adhesion is prolonged, the infected cell can stably anchor target cell membranes at the surface of infected cells to form virological synapses. In a process that depends on the presence of the cytoplasmic tail of MLV Env, the infected cell subsequently redirects de novo virus assembly to sites of cell-cell contact. Released viral particles then utilize the underlying F-actin flow of the target cell lamellipodium and lamellum to move towards the cell body of target cells. Here we present mechanistic insights into the establishment of adhesion and polarized assembly. We identify the motifs within the cytoplasmic tail of Env that are required to stabilize cell-cell contact and redirect assembly to sites of cell-cell contact. Moreover, we present the determinants within the viral Gag polyprotein that are required for trafficking to sites of cell-cell contact. Our data are consistent with a model of adhesion-induced polarity that allows the local nucleation of viral assembly at sites of cell-cell contact.

DYNAMICS OF VIRAL PROTEINS DURING THE NATURAL HIV-1 INFECTION – INSIGHTS FROM A BIARSENICAL-TETRACYSTEINE FLUORESCENT LABELING METHOD

Candida da Fonseca Pereira[1,2,3], Kate L Jones[1], Paula C Ellenberg[1], Valerie Vivet-Boudou[6], Roland Marquet[6], Iain Johnson[7], Johnson Mak[1,4,5]

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Natural HIV-1 infection occurs through receptor-mediated fusion. It has recently been suggested that HIV-1 enters target cells via receptor-mediated fusion with the endosomal membrane. After the virus enters the target cell it will need to reverse transcribe its viral genome. This is a dynamic process that involves a myriad of viral and cellular proteins that form the reverse transcription complex (RTC), facilitate viral cDNA synthesis and intracellular trafficking.

HIV-1 containing a green fluorescent protein-labeled viral protein R (GFP-Vpr) is recognized as the benchmark for the visualization of the RTC. However, tracking of Vpr alone is unable to reveal the dynamics of proteins within the viral uncoating complex. Furthermore, many HIV tracking studies used VSV-G-pseudotyped HIV-1 to facilitate entry into target cells through pH-sensitive fusion instead of the natural HIV-1 envelope receptor-mediated fusion. In an attempt to track HIV infections that rely on HIV envelope-mediated entry, we have created dual-labeled viruses where the C-terminus of the matrix protein is labeled with tetracysteine-FlAsH, the virion membrane is labeled with S-15-mCherry and/or the N-terminus of Vpr is labeled with mCherry or GFP. More importantly, our labeling of HIV-1 matrix with tetracysteine-FlAsH is highly specific without detectable background and does not impair viral reverse transcription or infectivity.

Our dual-tracking system has revealed two novel pieces of information: Firstly, approximately 80% of all HIV-1 virions that enter T-lymphocytes still contain virion membrane, which supports the notion that HIV enters its natural target cells via endocytosis. Secondly, the majority of the detected matrix proteins remain associated with the RTC throughout the reverse transcription process.

In conclusion, we have developed a dual-labeled fluorescent virus system that exclusively uses the natural HIV-1 envelope glycoproteins to infect T-lymphocytes and therefore lays the foundation to delineate the natural uncoating process of HIV-1. Furthermore, this system can also be applied to study the entry mechanism of other viruses.

NC-DEPENDANT LOCALIZATION OF HIV-1 GAG TO UROPODS IN POLARIZED T CELLS FACILITATES CELL-TO-CELL VIRUS TRANSFER

Nick Llewellyn[1], Akira Ono[2]
[1]University of Michigan, Cell and Molecular Biology, Ann Arbor, MI, 48103, [2]University of Michigan, Microbiology and Immunology, Ann Arbor, MI, 48103

T cells are highly motile and adopt a polarized morphology in lymph nodes where cell-to-cell transmission of HIV-1 is likely frequent. However, little is known about the HIV-1 life cycle in polarized T cells. Polarized T cells form two ends, the leading edge at the front and a protrusion called a uropod at the rear. Using multiple uropod markers, we determined that HIV-1 Gag localizes to the uropod in polarized T cells. Infected T cells formed contacts with uninfected target T cells preferentially via HIV-1 Gag-containing uropods compared to leading edges that lack plasma-membrane-associated Gag. Furthermore, a myosin light chain kinase inhibitor, which disrupts uropods, reduced virus particle transfer from infected T cells to target T cells. These results suggest that Gag-laden uropods enhance cell-to-cell transmission of HIV-1. Although cell-to-cell virus transfer required Env-CD4 interactions, we determined that Env, which is known to interact with Gag, is dispensable for Gag localization to the uropod. Interestingly, we observed that Gag copatches with antibody-crosslinked uropod markers even in non-polarized cells, suggesting an association of Gag with uropod-specific microdomains that carry Gag to uropods. Consistent with this possibility, lateral movement of Gag-containing patches to uropods was observed in live cell imaging of polarizing T cells. Finally, we determined that localization of Gag to the uropod depends on higher-order clustering driven by its NC domain, which uses RNA as a scaffold. Taken together, these results support a model in which NC-dependant Gag accumulation to uropods establishes a preformed platform that facilitates HIV-1 cell-to-cell transmission upon uropod contact with target cells.

SPREAD OF HIV THROUGH T CELL VIROLOGICAL SYNAPSES ENHANCES MULTIPLICITY OF INFECTION

Armando Del Portillo[1], Joseph Tripodi[2], Amanda LeBlanc[2], Vesna Najfeld[2], David N Levy[3], Benjamin K Chen[1]

[1]Mount Sinai School of Medicine, Division of Infectious Disease, Department of Medicine, New York, NY, 10029, [2]Mount Sinai School of Medicine, Division of Hematology and Oncology, Department of Medicine, New York, NY, 10029, [3]New York University College of Dentistry, Basic Science and Craniofacial Biology, New York, NY, 10010

HIV can infect cells through a cell-free intermediate or can be directly passed from an infected cell to an uninfected recipient cell. Cell-to-cell infection has been shown to be a more efficient route of spread in vitro. Our recent studies have revealed that large quantities of virus are internalized into recipient cells through T cell virological synapses. The amount of virus transferred can be thousands of fold greater than that achieved by cell-free virus. Here, we examined if cell-cell infection leads to high rates of simultaneous infection by multiple proviruses.

To measure the frequency of multiply infected cells following cell-free or cell associated infection, we infected with combinations of fluorescent protein-marked HIV proviruses, where dual or triple color infected cells provide a measure of coinfection. In our studies, we titrated cell-free and cell-associated infections over the same infection rates. Cell-free virus was harvested from cells expressing two different HIV proviruses, and donor cells were generated by cotransfection of two or three different HIV proviruses. When cell-free virus was titrated onto target cells, the coinfection rate was dependent on the square of the overall infection rate, and approached zero at low infection rates. However, when donor cells were titrated onto target cells, the coinfection rate was high at high infection rates, but remained at a set percentage that did not approach zero at low infection rates. To directly measure proviral copy number in cells, we performed fluorescence in situ hybridization (FISH) on cells infected by cell-free versus cell-associated virus and observed higher numbers of proviral DNA per cell following cell-cell infection.

We conclude that cell-free virus can be titrated to levels where coinfection does not occur, but cell-associated infection does not readily approach zero. The persistance of multiply infected cells at extremely low cell-cell infection rates (e.g. <0.05%) may facilitate HIV recombination or favor the maintenance of the viral quasispecies observed in vivo. The study suggests that the unit of genetic inheritance during cell-cell infection is often greater than one genome and has implications for modeling HIV evolution, development of drug resistance, immune escape and phenotypic variation.

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HIV CO-OPTS TROGOCYTOSIS TO TRAFFIC FROM DENDRITIC CELLS TO T CELLS DURING TRANS-INFECTION

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Dendritic cells (DCs) bind and transfer intact HIV to CD4 T cells by forming an "infectious synapse," where the bound virus is recruited to the site of cell contact along with CD4 and chemokine receptors on the T cell surface, providing an efficient conduit for virus entry. Activation of the DCs by bacterial LPS markedly enhances infectious synapse formation and trans-infection. We have previously shown that HIV is concentrated within a non-endosomal, surface accessible "pocket" within activated DCs and that individual virions can traffic from the pocket and rapidly fuse into the T cell at the infectious synapse. Here we demonstrate that activated DCs exhibit greatly decreased capacity to endocytose and process HIV peptides for antigen presentation, suggesting that there is a dynamic balance between trans-infection and antigen processing in dendritic cells. Careful examination of the synapses revealed that in a subset of conjugates, T cell surface markers co-localized with concentrated HIV within the DCs and appeared to be connected to the T cells by thin membrane extensions. Strikingly, T cell surface antigens were found within the HIV pocket in 30-40% of DCs even in the absence of interacting T cells. This transfer of surface markers between cells, known as "cell nibbling" or trogocytosis, occurred with similar efficiency in co-cultures with CD8 T cells and was largely blocked by anti-LFA-1 antibodies. Interestingly, the majority of CD4 T cells contained 1-2 virions per cell after 30 minutes of co-culture, whereas little or no viral transfer into CD8 T cells was observed. Our data suggests that LPS activation induces endocytic downregulation and HIV sequestration within the pocket-like structure. Contact with T cells results in surface invasion into the pocket and LFA-dependent transfer of T cell markers into the DC. If CD4 is present on the T cell membrane, HIV can exit the pocket and enter the T cell at the infectious synapse. Therefore T cells may play an important role in "fishing" HIV out of the pocket during trans-infection.

GAG TARGETING TO GLYCOSPHINGOLIPID-ENRICHED MICRODOMAINS PLAYS AN IMPORTANT ROLE IN DENDRITIC CELL-MEDIATED HIV-1 CAPTURE AND TRANSFER.

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HIV-1 has exploited dendritic cells (DCs) to promote their transmission to T cells. We recently reported that glycosphingolipids (GSLs) on virus particle membrane play an important role in DC-mediated virus capture and the subsequent virus transmission. HIV-1 is believed to bud from GSL-enriched lipid microdomains on the plasma membrane. Thus, we hypothesized that HIV-1 particles obtain determinants for DC-mediated capture by targeting Gag to GSL-enriched microdomains for release. To test this hypothesis, we introduced an in frame deletion or point mutations in matrix (MA) domain of HIV-1 Gag to potentially alter the site of virus budding, and tested the ability of DCs to capture and transfer mutant and wild type (WT) Gag-eGFP virus-like particles (VLPs) and infectious virus particles.

In contrast to WT-Gag-eGFP that was predominantly found at the plasma membrane in transfected cells, the Gag-eGFP mutants containing deletion (ΔMA) or point mutations (29/31KE) in MA were predominantly distributed at an intracellular compartment, indicating that modification of MA altered the VLP budding site. Though efficiency of VLP release was unaffected, ΔMA-VLPs contained significantly less GM3 than WT-VLPs, suggesting the ΔMA variant budded from non-GSL-enriched membranes. Interestingly, mature DCs could capture less ΔMA- and 29/31KE-VLPs than WT-VLPs (>80% reduction). The effect of MA-deletion on infectious virus budding was also confirmed by EM and IF and observed to occur mainly from $CD63^+$ $Lamp\text{-}1^+$ intracellular compartments. Importantly, the ability of mature DCs to capture NL43ΔMA virus was dramatically inhibited (~10% of the wild type NL43). Although truncating the cytoplasmic tail of gp41 (ΔCT) allowed the ΔMA variant to incorporate gp120 into particles similar to levels observed with parental NL43ΔCT, and rescued virus infectivity, the NL43ΔMAΔCT remained poor for capture by DCs and was severely attenuated for DC-mediated transfer to T cells, arguing that Env-independent capture mechanism is crucial for the ability of HIV-1 to access the DC-dependent *trans* infection pathway.

These results suggest that HIV-1 particles acquire determinants which are specifically recognized by DCs when Gag proteins are targeted to the GSL-enriched microdomains in the plasma membrane for release. Furthermore, these findings may explain in part why HIV-1 buds predominantly from the plasma membranes rather than from the intracellular membranes even though such intracellular virus budding sites might be better protected from antiviral agents such as neutralizing antibodies.

EVIDENCE FOR SEQUENCE VARIATION IN XMRV

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Viral genomic sequences of the human gammaretrovirus xenotropic murine leukemia virus-related virus (XMRV) have been detected in prostate cancer tissue and in the peripheral blood mononuclear cells (PBMCs) of individuals with chronic fatigue syndrome, as well as a small percentage of normal blood donors. However, several laboratories have been unable to detect XMRV sequences in prostate cancer tissue or PBMCs from CFS patients by DNA PCR. These differences could be due to a number of factors including geographical differences in the distribution of XMRV, differences in the assays used, or the presence of variants of XMRV in different populations.

The XMRV sequences published to date suggest that this virus has very little sequence diversity. We recently initiated studies to characterize XMRV from a large number of infected individuals. Peripheral blood from individuals with CFS or prostate cancer were examined, and more than 35 antibody positive individuals in each group were identified. Initially, nested PCR and RT-PCR for env was performed on DNA and RNA isolated from PBMCs from some of these individuals, using previously published primers. Env sequences were amplified from several of the samples; the sequences were identical or nearly identical to what had been previously reported for XMRV. However, for the majority of the samples, we were unable to amplify env sequences, even in samples from which virus had been isolated.

In contrast, gag sequences could be amplified from nearly all the samples, suggesting that there might be more variability in XMRV that previously reported. In addition, preliminary studies suggested that XMRV sequences directly amplified from PBMCs in patient samples differed from sequences amplified after viral spread following ex vivo culture.

We have now isolated RNA from unactivated PBMCs, and from PBMCs activated and cultured ex vivo, from more than 30 XMRV positive individuals and have amplified gag sequences using nested RT-PCR. The samples are currently being sequencing to determine extent of variability in these samples.

HIV-1 IN MUTANTS DEFECTIVE FOR INI1/HSNF5 BINDING ARE DEFECTIVE FOR POST-ENTRY EVENTS OF HIV-1 REPLICATION

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INI1/hSNF5 (Integrase interactor 1) is incorporated into HIV-1 virions by binding to IN. Transdominant mutants of INI1 inhibit HIV-1 replication and virions produced in INI1-/- MON cells are defective for infectivity. INI1 and IN interact with SAP18 and recruit HDAC1 complex into virions, which is required for early post-entry events.

To further understand the role of INI1 and associated SAP18-HDAC1 complex, we isolated a panel of IN mutants defective for INI1 binding using reverse yeast two-hybrid system. INI1 interaction defective mutations of IN were mapped on solved crystal structures of IN and mutations of residues with high surface accessibility on IN (S147G, Q137R, K71R, H12Y, K111E, D202G) were further characterized using virological assays. The panel of IN* mutants were incorporated into HIV-1NL4-3 and tested for infectivity in CEM-GFP cells. GFP expression and virion-associated p24 and RT activity were scored in a 16 day infection cycle. ALL mutants (including the previously reported K71R) were defective for replication compared to wild type in CEM-GFP cells. Two distinct classes of INI1-interaction defective IN* mutants--those that were highly defective (Q137R, H12Y, D202G) or partially defective (K71R, S147G, K111E) for replication were noted. The mutants were then incorporated into HIV-Luc and tested for their effect on early events. Consistent with the results of multiday replication assay, highly defective mutants were 10-100 fold defective and partially defective mutants were 2-5 fold defective in the HIV-Luc assay. Western blot analysis of mutant virions revealed the stable expression of all viral proteins including mutant INs except for highly defective H12Y, which showed a processing defect and ~5 fold reduction in virion associated IN. Analysis of early post entry stages of replication indicated that highly defective mutants were blocked at early and late reverse transcription, whereas partially defective mutants were not defective in these assays.

The above analysis indicated a block at or prior to reverse transcription in highly defective mutants, similar to a block we noted when HIV-1 virions were produced in INI1-/- cells or when defective HDAC1 mutant was incorporated into virions. Currently, we are analyzing the ability of these mutants to interact with RT, INI1 and/or SAP18 to determine the mechanism of block at or prior to reverse transcription. These studies will shed light on the role of INI1 and the associated SAP18-HDAC1 complex during HIV-1 replication.

A $tRNA^{Lys3}$-LIKE ELEMENT IN THE HIV-1 GENOME 5´-UTR ASSISTS IN PRIMING OF REVERSE TRANSCRIPTION

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A wide variety of transposable elements and viruses rely on tRNAs and tRNA-like structures during their life cycles. To initiate reverse transcription, HIV-1 anneals human $tRNA^{Lys3}$ to the complementary primer binding site (PBS), and cDNA synthesis begins from the 3´-OH of the tRNA. We have previously shown that human lysyl-tRNA synthetase (LysRS) is specifically incorporated into HIV-1 by the Gag protein and that this interaction is critical for $tRNA^{Lys3}$ packaging. Herein, we describe a novel conserved $tRNA^{Lys3}$-like element (TLE) in the 5´-UTR of the HIV-1 genome proximal to the PBS. Using electrophoretic mobility shift assays and fluorescence anisotropy binding studies, we show that LysRS interacts with the TLE, which mimics the anticodon domain of $tRNA^{Lys3}$, whereas human TrpRS and ProRS do not. The affinity of LysRS for short (23 nt) HIV-1 genome-derived RNA hairpins containing the TLE is similar to that of in vitro transcribed 76-nt $tRNA^{Lys3}$ ($K_d \sim 90$ nM). Human LysRS recognizes tRNALys isoacceptors via a specific interaction with the anticodon (UUU in $tRNA^{Lys3}$ and CUU in $tRNA^{Lys1,2}$). Mutation of the corresponding UUU motif in the TLE to AAA or CCC greatly reduces the LysRS-TLE interaction ($K_d \geq 2.5$ and 8 uM, respectively). Longer 330-nt RNAs derived from the 5'-UTR compete even more effectively for LysRS binding to a $tRNA^{Lys3}$ anticodon stem-loop mimic than $tRNA^{Lys3}$, suggest that in addition to the U-rich motif in the TLE, other sequence elements in the 5´ UTR contribute to preferential binding of LysRS to the HIV-1 genome. 293T cells transfected with virus containing TLE mutations show no change in the level of packaged $tRNA^{Lys3}$ but result in a 70% decrease in the amount of $tRNA^{Lys3}$ placed onto the genome. Taken together, these new findings suggest that the HIV-1 genome uses molecular mimicry of $tRNA^{Lys3}$ to facilitate primer release from LysRS and placement onto the PBS.

DUPLEX STRUCTURAL DIFFERENCES AND NOT 2'-HYDROXYLS EXPLAIN THE MORE STABLE BINDING OF HIV-RT TO RNA-DNA VS. DNA-DNA

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Human immunodeficiency virus reverse transcriptase (HIV-RT) must interact with RNA-RNA, RNA-DNA and DNA-DNA duplexes to complete reverse transcription. Interestingly, binary complexes between HIV-RT and RNA-DNA are much more stable than those with DNA-DNA. The most likely explanation(s) for this are: (1) RNA-DNA and DNA-DNA duplexes obtain H-form (structure between A-form (RNA-RNA duplex structure) and B-form (DNA-DNA duplex structure) but closer to the former) and B-form structures, respectively, and RT may bind more stably to H-form; (2) Crystal structures of RNA-DNA and DNA-DNA with RT have many similarities but a major difference is several additional interactions between RT amino acids and 2'-hydroxyls of the RNA template. This suggests a possible role for these interactions in stabilizing binding. Previously we showed that only 5 base pairs of RNA-DNA hybrid imbedded in a larger DNA-DNA duplex were required to mimic the stable binding of a complete RNA-DNA duplex, provided that the RNA-DNA was position in the polymerase active site (Bohlayer and DeStefano, 2006, *Biochemistry* 45:7628-38). Here, we present new results indicating that only 2 of the 5, the -2 and -4 RNA nucleotides (-1 hybridized to the 3′ recessed DNA base), are required to mimic RNA-DNA binding, and even a single RNA nucleotide at the -4 position confers significantly greater stability than DNA-DNA. Replacing 2′- hydroxyls on pivotal RNA bases with 2′-O-methyls did not affect stability, indicating that interactions between hydroxyls and RT amino acids do not stabilize binding. RT's K_d (k_{off}/k_{on}) for DNA-DNA and RNA-DNA were similar, although k_{off} differed almost 40-fold, suggesting a faster k_{on} for DNA-DNA. Avian myeloblastosis and Moloney murine leukemia virus RTs also bound more stably to RNA-DNA, but the difference was less pronounced than with HIV-RT. We propose that the H- vs. B-form structures of RNA-DNA and DNA-DNA, respectively, allow the former to conform more easily to HIV-RT's binding cleft, leading to more stable binding. Notably, crystal structures of HIV-RT with duplexes show that the 4 base pairs near the polymerase active site in the "primer-grip" (the region of RT most associated with substrate binding) region of RT obtain an A-form like structure while those in the connection and RNase H domains are closer to B-form. The H-form structure of RNA-DNA hybrids is also "A-form like" and may simply conform better than DNA-DNA for binding in the primer grip region. Biologically, the ability of RT to form a more stable complex on RNA-DNA may aid in degradation of RNA fragments that remain after DNA synthesis.

CONSERVATION AND MECHANISM OF ANTI-HIV-1 ACTIVITY OF TRIM 37

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There is strong experimental evidence that several members of the tripartite motif (TRIM) family of proteins possess antiretroviral activity. In particular, Trim5alpha, Trim22, Trim19, and Trim32 have all been shown to possess anti-HIV-1 activity. We have identified another member, Trim37, as having the ability to potently restrict HIV-1 replication. To date, our data supports a model in which Trim 37 interacts with HIV-1 reverse transcriptase (RT), is incorporated into HIV-1 particles, and interferes with reverse transcription. In our current studies, we have investigated whether Trim 37 has been under positive selection and whether the antiretroviral activity is functionally conserved. Our phylogenetic analyses have implicated that Trim 37 has been under positive selection, which supports its being associated with intrinsic immunity to retroviral replication. Functional analyses of Trim 37 from various mammalian species (i.e., various non-human primate species including African green monkey, bovine, and feline) have verified conserved antiretroviral activity. These studies provide insight into both the antiretroviral determinants of Trim 37 as well as implicate the ability of Trim 37 to interfere HIV-1 reverse transcription by reducing RT processivity.

DEVELOPMENT OF AN FV1 BINDING ASSAY.

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The restriction factor Fv1 confers resistance to Murine leukaemia virus (MLV), blocking progression of the viral life cycle after reverse transcription has occurred, but before integration into the host chromosome. It is known that the specificity of restriction is determined by both the restriction factor and the viral capsid (CA), but a direct interaction between Fv1 and MLV CA has not yet been demonstrated.

With the development of a novel method for in vitro polymerisation of MLV CA, it has now been possible to display a binding interaction between Fv1 and MLV CA. C-terminally His-tagged CA molecules were assembled on Ni-containing lipid-based nanotubes. Analysis of these tubes by electron microscopy showed that at the appropriate concentration of Ni a regular lattice is formed. Incubation with of these tubes with lysates from cells expressing Fv1 followed by centrifugation enabled binding to be demonstrated.

Comparison of binding data with existing restriction data confirms the specificity of the interaction. For example, product of the Fv1n allele bound CA from B tropic MLV but not N-MLV and was sensitive to mutations converting N-MLV to NR-MLV or mutations interfering with proper CA assembly. Further, an inactive Fv1 mutation (Int1) previously hypothesized to loose restriction activity as a result of mis-localization within the cell was shown to bind CA normally whereas a second mutation (in the Fv1 MHR), which is likely to interfere with Fv1 folding, did not. We are currently using this novel binding assay to further investigate the mechanism by which Fv1 restricts MLV infection.

CONTRIBUTION OF E3 UBIQUITIN LIGASE ACTIVITY TO HIV-1 RESTRICTION BY TRIM5A

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TRIM5α proteins from rhesus monkeys (TRIM5αrh) block human immunodeficiency virus (HIV-1) infection prior to reverse transcription. TRIM5αrh protein consists of a RING, B-box 2, coiled-coil and B30.2(SPRY) domains. The contribution of each independent TRIM5α domain to restriction has been extensively studied with the exception of the RING domain. The RING domain of TRIM5α contributes to the potency of retroviral restriction and possesses ubiquitylation activity. Substrate ubiquitylation starts by the formation of a covalent link between ubiquitin and enzyme-1 (E1), which transfers ubiquitin to the active site of E2. The ubiquitin conjugated-E2 interacts with an E3 ubiquitin ligase that transfer ubiquitin from the E2 to a lysine residue in the ubiquitylation substrate. The RING domain of TRIM5α possesses two functional surfaces important for E3 ubiquitin ligase activity: 1) the E2 binding surface, and 2) the association surface, which in many cases improves the catalytic transfer of ubiquitin. Because the contribution of E3 ubiquitin ligase activity to restriction is not understood, we tested the hypothesis that the E3 ubiquitin ligase activity of the RING domain modulates HIV-1 restriction by TRIM5αrh. To test our hypothesis, we correlated the E3 ubiquitin ligase activity of a panel of TRIM5αrh RING domain variants to the ability of these mutants to restrict HIV-1. These results established a role for E3 ubiquitin ligase activity in HIV-1 restriction by TRIM5α.

TRIM5 IS BOUND TO THE 19S REGULATORY PARTICLE OF THE 26S PROTEASOME.

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TRIM5 proteins restrict retrovirus life cycle at least at two stages: by targeting early post-entry event(s) that lead to reverse transcription of the viral genome, which requires functional 26S proteasome, and by interfering with transport of the viral preintegration complex to the nucleus. To address the underlying mechanisms we performed comprehensive proteomic analyses of TRIM5 associated cellular proteins from cell lines stably expressing rhesus monkey TRIM5. Strikingly, we identified 26 out of 31 subunits of 26S proteasome in highly purified TRIM5 preparations. This demonstrated that TRIM5 tightly associates with 26S complex, but was surprising in that 26S complex normally dissociates into its 19S regulatory and 20S catalytic sub-complexes under the purification conditions used. This probably reflects TRIM5 degradation since such a behavior is expected of stalled 26S proteasome-substrate complexes. Next we addressed the roles of TRIM5 poly-ubiquitylation. A systematic scan of the TRIM5 molecule with lysine-to-arginine substitutions revealed that at most only 4 lysines located in the SPRY domain are required for TRIM5 to restrict infection, and these lysines are not the sites of poly-ubiquitylation. We also analyzed the ability of a mutant TRIM5 molecule (K74R) that retains only 6 lysines in its RING domain and is not poly-ubiquitylated to bind 26S proteasome. TRIM5 K74R stably and specifically associated with 26S proteasome, as it precipitated the complete 26S proteasome complex under conditions that stabilize the 26S complex. Strikingly, and in contrast to wild-type TRIM5, K74R precipitated only the 19S regulatory sub-complex under conditions that favor 19S dissociation from 20S, thus indicating that it docks specifically to the 19S regulatory sub-complex. The spectrum of 19S subunits found associated with K74R suggests that it docks to 19S probably via its base, similar to other proteins that modulate proteasome function, and, importantly, confirms that K74R binding to 19S is not mediated by TRIM5 poly-ubiquitylation, because the 19S subunits that serve as polyubiquitin receptors were not found. Our findings indicate that TRIM5, besides being degraded by the proteasome, also stably associates with the 19S regulatory sub-complex by a poly-ubiquitylation-independent mechanism. We hypothesize that via its binding to the 19S sub-complex TRIM5 recruits 26S proteasome to the restricted viral capsid, and that this physical interaction with 19S sub-complex is important for TRIM5's full ability to restrict retrovirus infection.

P62/SEQUESTOSOME1 ASSOCIATES WITH AND SUSTAINS THE EXPRESSION OF THE RETROVIRAL RESTRICTION FACTOR TRIM5A

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TRIM5 proteins mediate a potent block to the cross-species transmission of retroviruses, the most well known being the TRIM5α protein from rhesus macaques, which potently inhibits human immunodeficiency type-1 (HIV-1) infection. This restriction occurs at an early stage in the replication cycle and is mediated by the binding of TRIM5 proteins to determinants present in the retroviral capsid. TRIM5α, as well as other TRIM family proteins, have been shown to be regulated by interferons (IFN). Here we show that TRIM5α associates with another IFN-induced gene, Sequestosome-1/p62 (p62). p62 plays a role in several signal transduction cascades that are important for maintaining the anti-viral state of cells. Here we demonstrate that p62 localizes to both human and rhesus TRIM5α cytoplasmic bodies, and fluorescence resonance energy transfer (FRET) analysis demonstrates that these proteins closely associate in these structures. When p62 expression was knocked down via siRNA, the number of TRIM5α cytoplasmic bodies and TRIM5α protein expression was reduced in cell lines stably expressing epitope tagged versions of TRIM5α. In accordance with this data, p62 knockdown resulted in reduced TRIM5α mediated retroviral restriction in cells expressing epitope tagged TRIM5α or expressing endogenously expressed human TRIM5α. p62 may therefore operate to enhance TRIM5α mediated retroviral restriction, enhancing the antiviral state of cells following IFN treatment.

ESCAPE FROM TRIM5A-MEDIATED SUPPRESSION DURING CROSS-SPECIES TRANSMISSION AND EMERGENCE OF PRIMATE LENTIVIRUSES

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TRIM5α blocks retroviral replication when expressed in otherwise susceptible target cells. Based on *in vitro* assays and phylogenetic comparisons, it is widely assumed that TRIM5α prevents cross-species transmission of retroviruses in nature, and that spread of pathogenic viruses in a new host, when it occurs, drives protective alleles of TRIM5 to fixation. Simian immunodeficiency viruses of sooty mangabeys (SIVsm) are the source of multiple cross-species transmission events, giving rise to HIV-2 in humans, SIVmac in rhesus macaques, and SIVstm in stump-tailed macaques. We found that SIVsm, SIVstm, HIV-1 and HIV-2 were sensitive to multiple alleles of rhesus macaque TRIM5, including TRIM5-CypA, while SIVmac was resistant to all alleles tested. We also analyzed experimental cross-species transmission of SIVsm into rhesus macaques, the most widely used species in preclinical AIDS vaccine research, and found a significant correlation between TRIM5 genotype, viral replication, and emergence of allele-specific escape mutations in the N-terminal domain of capsid. Unexpectedly, transmission occurred even in individuals bearing restrictive TRIM5 genotypes, resulting in attenuation of replication rather than an outright block to infection. Using phylogenetic analyses and site-directed mutagenesis experiments, we identified two adaptive changes in the SIVmac239 capsid that render it resistant to specific alleles of rhesus TRIM5.

Thus, TRIM5α suppresses viral replication *in vivo*, exerting selective pressure during the initial stages of cross-species transmission. However, restriction by TRIM5 alone may be insufficient to prevent cross-species transmission of primate lentiviruses, and once a virus has adapted to a host population (as is the case with HIV-1 in humans and SIVmac in macaques), the role of restriction in limiting spread diminishes relative to genes of adaptive immunity.

CONFORMATIONAL DIVERSITY ENABLES TRIMCYP TO RESTRICT MULTIPLE VIRUSES

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One of the central principles of biochemistry is that the amino acid sequence of a protein determines a single three-dimensional structure. This fidelity of structure is crucial to maintaining specificity of function in proteins such as enzymes. In innate immunity, however, this limits the usefulness of antiviral proteins due to the tremendous diversity of viruses and their capacity to evolve. We have discovered that the lentiviral restriction factor Rhesus TRIMCyp (RhTC) has addressed this problem by evolving the ability to isomerise between multiple conformations, each of which targets a different virus from a distinct lentiviral lineage. This multispecificity has evolved following retrotransposition of a duplicated (CypA) domain and fusion with the tripartite domains of TRIM5. We have used NMR dynamics to show that subsequent mutation and selection of two residues in the CypA domain have released a constrained active-site loop, leading to dynamic sampling of conformational space. Determination of complexed crystal structures reveal how this modified RhTC CypA domain selects a closed loop conformation to bind particular strains of HIV-1 and an open loop conformation to bind HIV-2. Although Linus Pauling predicted ‘conformational diversity’ over 70 years ago, to our knowledge this is the first example of such a mechanism being used to expand protein function. Conformational diversity may functionally augment genetic diversity and be a general host response to the selective pressure exerted by lentiviruses.

P21$^{CIP1/WAF1}$ CONTROLS HIV-1 REPLICATION IN PRIMARY HUMAN MACROPHAGES AND CD4+ T LYMPHOCYTES

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The cyclin-dependent kinase inhibitor p21$^{Cip1/Waf1}$ has been involved in the resistance of hematopoietic stem cells to HIV-1 infection, by inhibiting viral DNA integration. We showed that the induction of p21 expression in monocyte derived macrophages (MDM) by immune complex (IC)-aggregation of FcγR or by the histone deacetylase inhibitor MS-275 was associated to a strong and dose-dependent inhibition of HIV-1 replication. The levels of reverse transcripts and integrated proviruses in one-round infections with VSV-G pseudotyped HIV-1 (NL4.3$_{VSV-G}$) were decreased by p21 induction. Gene knockdown of p21 increased both HIV-1 reverse transcripts and integrated forms in unstimulated MDM and rescued the defect of HIV-1 replication in FcγR-activated cells. To confirm that p21 is specifically involved in the inhibition of HIV-1 infection, we transfected MDM with GST-tagged and HA-tagged p21 expression vectors. The overexpression of p21 reduced HIV-1 replication by 96% and 93% respectively. These results indicate that p21 inhibits HIV-1 replication blocking early post-entry steps of viral life cycle. We next asked whether p21 could play an antiviral activity in other cellular targets of HIV-1. We knocked down p21 expression in primary CD4+ T cells and infected them with NL4.3$_{VSV-G}$. The depletion of p21 produced a significant increase (63%) of HIV-1 replication compared to CD4+ T cells transfected with an irrelevant siRNA, indicating that p21 exerts an inhibitory activity on HIV-1 infection in these cells.
However, p21 was previously reported to enhance HIV-1 replication in chronically infected MDM. Thus, we asked whether p21 may differently affect post-integrative steps of HIV-1 replication. We transfected MDM with an HIV-1 NL4.3 coding vector and we studied the effect of IC-mediated activation on LTR-driven transcription. HIV-1 transcription was increased of 3.2-fold in IC-activated MDM compared to unstimulated MDM.
Our data show that p21 acts as an intrinsic barrier to HIV-1 replication in the major target cells of HIV-1. In addition, our results also suggest that p21 may play a dual role in HIV-1 infection of macrophages, inhibiting the preintegrative steps of the incoming virus and enhancing HIV-1 gene transcription after viral integration. Pharmacological compounds, such as HDACi, that are currently studied as adjuvant to highly active antiretroviral therapy to eradicate HIV-1 reservoirs, also induce p21 expression and might thus exert a double therapeutical role; they may induce HIV-1 expression from latently infected cells while limiting HIV-1 reinfection of lymphoid and myeloid target cells.

CYTOSKELETAL REGULATORY PROTEINS TALIN 1 AND VINCULIN DOWNREGULATE RETROVIRAL INFECTION

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Post-entry transport mechanisms of retroviruses are currently not well understood but it is believed that the host cytoskeleton must play an integral role and it has been shown that productive human immunodeficiency virus type 1 (HIV-1) infection is dependent on functional actin dynamics. Here we show that the actin-interacting cytoskeletal regulatory proteins, talin 1 and vinculin function during retroviral infection. Talin 1 and vinculin directly interact with each other and are involved in the linkage of integrins to the actin cytoskeleton. To assess the involvement of talin1 and vinculin in retroviral infection we have used siRNA to transiently knockdown expression of each gene followed by infection with pseudo-typed HIV-1 virus and measured infection levels. Two independent infection assays have confirmed that human cells with either talin 1 or vinculin knocked down become more susceptible to pseudo-typed HIV-1 and simian immunodeficiency virus (SIV) infection. Additionally, over-expression of talin 1 or vinculin decreases susceptibility to HIV-1 infection. Vesicular stomatitis virus (VSV-G) and amphotropic- pseudotyped HIV-1 are blocked by talin 1 or vinculin indicating that the block to infection is post-entry. Viral block assays were performed to examine where in the retroviral life cycle talin 1 and vinculin block infection. Our data suggests that both talin 1 and vinculin down-regulate HIV-1 infection post-entry but before the initiation of reverse transcription.

THE PRINCIPAL FUNCTION OF THE HIV-1 CENTRAL DNA FLAP IS DEFENSE AGAINST APOBEC3 PROTEIN RESTRICTION

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HIV-1 and certain other retroviruses initiate plus strand synthesis in the center of the genome as well as at the standard retroviral 3' polypurine tract. This peculiarity of reverse transcription results in a central DNA "flap" structure that has been of controversial functional significance. We mutated both HIV-1 flap-generating elements, the central polypurine tract (cPPT) and the central termination sequence (CTS). To avoid an ambiguity of prior studies, we did so without changing integrase coding. Single cycle infection by 293T cell transfection-produced virus was unaffected in all target cells tested. Spreading HIV-1 infection was also normal in most T cell lines and in fibroblast cell lines, and flap-mutant viruses replicated equivalently to wild type in nondividing cells, including macrophages. However, spreading infection of flap-mutant HIV-1 was selectively impaired in Vif non-permissive cells (HuT78, H9, primary human PBMCs), suggesting APOBEC3G (A3G) restriction. Single cycle infections confirmed that vif-intact flap-mutant HIV-1 is restricted by producer cell A3G. Combining vif and cPPT-CTS mutations increased A3G restriction synergistically. Moreover, RNAi-mediated knockdown of A3G in HuT78 cells released the block to flap-mutant HIV-1 replication. Flap-mutant HIV-1 accrued markedly increased A3G-mediated G to A hypermutation compared to wild-type HIV-1 (a full log10 in a single cycle in the 0.4 kb downstream of the mutant cPPT). We conclude that the triple-stranded DNA structure, the flap, is not the main consequential outcome. The salient functional feature is central plus strand initiation, which functions as a second line of defense against the antiviral effects of A3 proteins that survive producer cell degradation by Vif.

HIV-1 INTERACTS WITH THE CYTOSKELETON VIA ITS CAPSID DURING ITS TRANSPORT TOWARDS THE NUCLEAR COMPARTMENT

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Although HIV-1 capsid disassembly (defined as uncoating) can occur precociously following cell entry, for instance upon TRIM5α restriction, evidence suggests that this cannot lead to productive infection. Indeed, successful uncoating is thought to occur much later, after reverse transcription and immediately prior to nuclear import. Our previous work suggests that HIV-1 uses both microtubule and actin networks to reach the nuclear membrane and that uncoating occurs at the nuclear pore. We therefore hypothesised that the capsid is the viral component that interacts with the cytoskeleton during its transport towards the nuclear compartment. To identify the cytoplasmic elements that interact with HIV intracellular complexes during cytoplasmic transport, we carried out a yeast-two-hybrid screen against either whole CA or helices 4-7, which include the interaction domain for cyclophilin A. We thus identified six CA-interaction partners, four of which are involved in cytoskeletal transport networks and two in nuclear transport. We then verified these candidates for interaction with native capsid cores, isolated directly from cell-free particles stripped of viral envelope. Furthermore we used RNA interference to evaluate which cellular interaction partners of viral CA are required or essential for HIV infection. This work sheds new light on the functional interplay between HIV-1 transport towards the nucleus and timely uncoating.

IS TRANSPORTIN-SR2 MEDIATED NUCLEAR IMPORT OF HIV MEDIATED BY INTEGRASE OR CAPSID?

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Transportin-SR2 (TRN-SR2) is a human karyopherin that imports SR-rich proteins from the cytoplasm into the nucleus. We identified TRN-SR2 as a binding partner of HIV-1 integrase (IN) and validated TRN-SR2 as an important cellular cofactor for the nuclear import of the HIV-1 pre-integration complex (PIC). In contrast, recent reports have suggested that not IN but the viral capsid (CA) protein determines TRN-SR2 dependency during viral infection. The HIV-1 N74D CA mutant was shown to be insensitive to TRN-SR2 knockdown. These contradictory findings prompted us to study in more detail the specific roles of integrase and capsid in TRN-SR2 mediated nuclear import of the HIV-1 PIC.
By infection of HeLaP4 cells depleted for TRN-SR2 and control cells with VSV-G pseudotyped single round HIV-1 reporter viruses carrying wild type or N74D mutant capsid proteins we confirm that the N74D CA mutation abrogates the inhibition of infection by TRN-SR2 knockdown. However, when TRN-SR2 knockdown and control cells were infected with viruses carrying the HIV-1 envelope, the HIV-1 N74D CA mutant regained sensitivity to TRN-SR2 knockdown. Much alike VSV-G, the viral envelopes of Ebola and rabies viruses that mediate entry of target cells via endocytosis rendered the HIV-1 N74D CA mutant insensitive to TRN-SR2 knockdown. Together with a lack of evidence for a direct interaction of TRN-SR2 with CA, this demonstrates that the TRN-SR2 independent phenotype displayed by the VSV-G pseudotyped HIV-1 N74D CA mutant is not dependent on TRN-SR2 interacting with CA but rather on the mode of viral entry and/or trafficking of the viral particle in the target cell.
We compared the impact of TRN-SR2 knockdown on transduction efficiency by different lentiviral and retroviral vectors and analyzed the direct interactions between the viral integrase proteins corresponding to these vectors and TRN-SR2. We show that TRN-SR2 binds readily to HIV-1, HIV-2, EIAV, MLV and RSV integrase in the alphascreen binding assay. Using deletion mutants and single amino acid substitutions we were able to pinpoint in HIV-1 integrase the TRN-SR2 binding site in *in vitro* and cell culture experiments.
Together these data support our hypothesis that IN plays a direct role in the nuclear import of the viral PIC. By playing a role in uncoating which precedes the interaction between IN and TRN-SR2, capsid has an indirect effect on the process of nuclear import.

REQUIREMENT FOR CELLULAR TRANSPORTIN 3 DURING THE EARLY PHASES OF LENTIVIRAL INFECTION AND VIRAL DETERMINANTS INVOLVED

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The early phases of lentiviral infection are carried out through a complex set of interactions established between viral and cellular proteins at basically all steps of the viral life cycle, from virus cell entry to vDNA integration. Among such cellular factors, a previous genome wide screen identified transportin 3 (TNPO3 or TRN-SR2) as a factor modulating the early steps of the HIV-1 life cycle, intervening following viral DNA synthesis at or immediately after nuclear import.
TNPO3 has been shown to interact directly with the viral Integrase protein (IN) and to be the cellular partner through which the latter mediates nuclear import of viral DNA. However, recent data seem to contradict this finding, indicating that the Capsid protein (CA), rather than IN mediates viral access to the nucleus via TNPO3.
To identify the viral and cellular determinants required for this rather unclear step of the viral life cycle, we have used lentiviral vectors derived from HIV-1 deleted or mutated for cis and trans viral elements alone or in combination (cPPT-CTS, Vpr, MA, CA and IN). Then, we have examined the effect that ablation of the expression of TNPO3 had on these mutants with respect to the early phases of infection. Our results indicate that among the viral elements examined here only the cPPT-CTS exerted a small, albeit measurable effect on the kinetics of viral DNA entry into the nucleus, but these effects were independent on TNPO3. When viruses presenting a complete deletion of IN were used to infect transportin 3 positive or knock down cells similar defects in infectivity were observed, indicating that IN is not the viral determinant required for the effect of TNPO3 on viral infectivity.
Next, mutations in CA were coupled to the IN deletion and the requirement for TNPO3 studied in cycling, as well as arrested cells in presence or absence of cytoskeleton inhibitors that may affect the virus ability to reach or cross the nuclear pore. These and other results on the relationship between nuclear import, transportin 3, CA and IN will be presented.

* Contributed equally to this work
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DISTINCT MOTIFS WITHIN THE TRANSPORTIN 3 CARGO BINDING DOMAIN ARE NECESSARY FOR LENTIVIRAL INFECTION

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To infect nondividing cells, the lentivirus preintegration complex (PIC) must transit through the nuclear pore into the nucleus. Two independent whole genome siRNA screens for host proteins that play a role in HIV-1 infection and a yeast two-hybrid screen for host proteins that bind to HIV-1 integrase (IN) identified TNPO3, a protein that transports cellular cargoes into the nucleus, as being necessary for HIV-1 nuclear import. These data suggest that TNPO3 transports a viral or cellular cargo whose nuclear import is directly or indirectly necessary for PIC nuclear import. We demonstrated that stable and transient knocked-down of TNPO3 blocked single-cycle HIV-1 and SIVmac reporter virus infection. Quantitation of reverse transcripts by qPCR showed that TNPO3 knock-down dramatically reduced HIV-1 and SIVmac 2-LTR circles, a marker for nuclear import, but had no effect on production of the viral cDNA. TNPO3 was also required by other lentiviruses, including EIAV and FIV, but not by the γ-retrovirus, MLV. Of these lentiviruses, SIVmac and EIAV were the most sensitive to TNPO3 knock-down and we are currently using HIV-1/SIVmac hybrid viruses to map the viral factors that contribute to these differing sensitivities to TNPO3 knock-down. In addition, we mapped functional domains of TNPO3 using a complementation assay in which stable TNPO3 knocked-down cells were transfected with a knock-down resistant TNPO3 construct containing silent mutations in the shRNA target site. We used this assay to show that the C-terminal cargo-binding domain of TNPO3 was critical for the restoration of SIVmac infection. Further mapping studies identified two distinct regions within the cargo-binding domain that were necessary for complementation. These mutant TNPO3 proteins maintained their nuclear localization, suggesting that their inability to restore SIVmac infection was the result of an inability to bind an as yet unidentified host or viral cargo. Although HIV-1 IN was reported to interact directly with TNPO3, repeated attempts to detect this and other interactions with HIV-1 proteins by coimmunoprecipitation from human cells were unsuccessful. We are currently using a proteomics approach to identify cellular TNPO3 cargo proteins that could be critical for lentiviral infection.

TNPO3 PROMOTES UNCOATING OF HIV-1 IN VITRO

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Uncoating, a poorly understood step in the HIV-1 life cycle, is defined as disassembly of the viral capsid following penetration of the core into the target cell. Previous results from our group have shown that mutations in CA that stabilize or destabilize the capsid are deleterious to infection, indicating that the stability of the capsid is optimized for executing the early phase of infection and that inefficient uncoating results in a postentry defect (Forshey et al., 2002). Furthermore, an HIV-1 CA mutant that undergoes inefficient uncoating leads to impaired nuclear entry, suggesting that the extent of uncoating is a critical determinant of nuclear import of the HIV-1 preintegration complex (Dismuke and Aiken, 2006). Though it seems likely that host factors regulate the uncoating process, to date no specific HIV-1 uncoating factor has been identified. Transportin 3/TRN-SR2 (TNPO3) is a karyopherin that was recently shown to promote HIV 1 infection (Brass et al., 2008). TNPO3 binds to IN and promotes nuclear import of the preintegration complex (Christ et al., 2008); however, a recent study identified CA as a major viral determinant of TNPO3-dependent retrovirus infection (Krishnan et al., 2010). Here we show that TNPO3 promotes uncoating of native HIV-1 cores in vitro. We also demonstrate the biological relevance of this finding using RNA interference. Depletion of TNPO3 resulted in an approximately 90% decrease in cellular permissiveness to wild-type HIV-1. By contrast, infection by CA mutants with hyperstable capsids was less dependent on TNPO3 expression. Collectively, our results suggest that TNPO3 facilitates infection by promoting HIV-1 uncoating following reverse transcription, thus facilitating translocation of the preintegration complex through the nuclear pore.

TRANSPORTIN 3 PROMOTES A NUCLEAR UNCOATING STEP REQUIRED FOR EFFICIENT HIV-1 INTEGRATION

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To replicate, HIV-1 must integrate into host chromosomes however little is known on the early events occurring post-nuclear entry but before integration. We have found evidence that HIV-1 must undergo a nuclear uncoating step to integrate. We have studied the behavior of HIV-1 in cells depleted of Transportin 3 (Tnp3) by transient or stable RNAi. HIV-1 infection efficiency was significantly lower in Tnp3 knock down (KD) cells compared to control cells. Viral DNA synthesis proceeded normally in KD cells. Production of 2LTR circular viral DNA forms was also unaffected in KD cells and fractionation experiments indicated that viral DNA accumulated into the nuclei of KD cells as efficiently as in control cells. However HIV-1 integration, measured by Alu-PCR, was defective in Tnp3 KD cells and fully accounted for the reduced infection. Pull-down assays showed that Tnp3 bound tRNAs and capsid proteins (CA) present in purified viral particles in a RanGTP dependent way. The C-terminus of Tnp3 was required for binding to both viral tRNAs and CA, suggesting that their binding is cooperative. The dependence on RanGTP strongly suggested that Tnp3 was an export factor for viral tRNAs and CA. The tRNA export activity of Tnp3 was confirmed in the permeabilized cell assay. To test if Tnp3 could also export CA, KD and control cells were infected with HIV-1 and the distribution of CA examined by Western blot following nuclear and cytoplasmic fractionation. Whereas significant amounts of CA were detected in the cytoplasm of control cells, little CA was detected in the cytoplasm of KD cells and the ratio of nuclear/cytoplasmic CA was greater in KD than control cells. The HIV-1 mutant bearing a single N74D change in CA was insensitive to Tnp3 KD and showed a similar ratio of nuclear/cytoplasmic CA in KD and control cells, supporting the link between export of CA and defective HIV-1 integration. We propose that tRNAs and some CA are retained in the HIV-1 RTC/PIC to promote its nuclear import, however these elements must be removed from the PIC at at stage after nuclear entry to allow efficient integration. Our results provide evidence for an unanticipated step in the HIV-1 life cycle that connects capsid and tRNA nucleocytoplasmic trafficking to HIV-1 integration.

CPSF6 RESTRICTION OF HIV

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HIV replication requires transport of nascent viral DNA and associated virion proteins, the retroviral preintegration complex (PIC), into the nucleus. Too large for passive diffusion through nuclear pore complexes (NPCs), PICs are dependent on cellular nuclear transport mechanisms and nucleoporins (NUPs), the NPC components that permit selective nuclear-cytoplasmic exchange. The mechanism of HIV PIC interaction with the NPC has not been defined. We identified a fragment of the cleavage and polyadenylation factor 6, CPSF6, as a potent inhibitor of HIV infection. When enriched in the cytoplasm, CPSF6 prevents HIV nuclear entry by targeting the viral capsid (CA). HIV harboring an N74D mutation in CA fails to interact with CPSF6 and evades the nuclear import block. We have mapped residues within CPSF6 necessary for HIV restriction. TRIM5, lacking viral targeting sequences, when N-terminally fused to 58 residues of CPSF6 restricts wild-type but not N74D HIV. Although CPSF6 restricts after reverse transcription, TRIM-CPSF6 prevents the initiation reverse transcription, providing further evidence for a direct interaction of CPSF6 with CA. Interestingly, resistance to CPSF6 alters HIV dependence on transporters and NUPs. Whereas wild-type HIV requires TNPO3 and NUP153, N74D HIV mimics feline immunodeficiency virus nuclear import requirements and is more sensitive to NUP155 depletion. Examination of primary and transformed cell types reveal different susceptibilities to wild-type vs. N74D HIV, suggesting transport factors may reciprocally act as tropism determinants. These findings reveal a remarkable flexibility in HIV nuclear transport and highlight CA as essential in regulating interactions with NUPs.

HIV-1 COUPLES NUCLEAR ENTRY PATHWAY WITH INTEGRATION SITE SPECIFICITY

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Lentiviruses can infect non-dividing cells by trafficking through the nuclear pore but the molecular details of this process remain unclear. Mutations of the viral capsid protein have been shown to impact on HIV-1 infectivity in non-dividing cells suggesting a central role for capsid in this process. Here we show that HIV-1 capsid directly interacts with the cyclophilin domain (Cyp) of the nuclear pore protein Nup358 with similar affinity and thermodynamic characteristics as it binds CypA. Furthermore, mutants that do not bind CypA do not bind Nup358. We have also determined the crystal structure of an HIV-1 N-terminal capsid domain in complex with the Nup358 Cyp domain, revealing how binding is mediated and why the Cyp antagonist cyclosporine fails to inhibit Nup358 Cyp-CA interaction. We demonstrate that the Nup358 Cyp domain actively catalyses *cis-trans* prolyl isomerisation in a model CypA peptide substrate suggesting that Nup358 might use this activity to liberate the pre-integration complex from the HIV 1 core at the nuclear pore. Reducing Nup358 levels using RNAi decreases the infectivity of HIV-1. Crucially, capsid mutants that do not interact with Nup358 Cyp, as measured by ITC, as well as mutants that are insensitive to Nup358 RNAi, are also less dependent on transportin 3 (TNPO3 or TRN-SR2), suggesting transportin 3 carries the HIV-1 PIC through the Nup358 nuclear pore. Finally, sequence integration site analysis suggests that entry via Nup358 determines integration site specificity. Nup358 RNAi insensitive mutants can use alternate pathways for nuclear entry but this directly affects site specificity. These observations suggest HIV-1 conserves CypA-binding to utilize a Nup358/transportin 3 pathway of nuclear entry and ensure integration into preferred sites in the genome that are optimal for replication *in vivo*.

NUCLEAR ENVELOPE PROTEOMIC ANALYSIS OF HIV-1-INFECTED T-CELLS IDENTIFIES FACTORS RESPONSIBLE FOR BLOCKING HNRNP SHUTTLING

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HIV-1 utilizes host proteins to its advantage at each step of its replication cycle. Consequently, recent investigations have aimed at identifying host cellular factors that are modified in expression, or whose functions are usurped during HIV-1 infection. In our previous work, we showed that HIV-1 induced cytoplasmic relocalization of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) was dependent on the nuclear export of the unspliced viral RNA, and on alterations in the localization and abundance of Transportin 1 and nucleoporin p62, respectively. This strongly suggested that, like other viruses, HIV-1 causes a block to nuclear import as a strategy to enhance its replication. In order to identify key compositional changes to the nuclear envelope (NE) by HIV-1, we isolated the NEs of T-cells at the peak of HIV-1 infection. We verified the purity of NEs by western and imaging analysis at each step of the fractionation procedure. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis identified 296 proteins, including core NE proteins, nucleoporins, transport receptors and shutting proteins. We found that 36% of proteins were modified in expression at the peak of viral production. Our LC/MS/MS results and western analysis supported our earlier work (i.e., hnRNPs, Transportin 1 and p62), and has provided additional insights into key proteins that may be responsible for the block in nuclear-cytoplasmic shuttling. Additionally, we identified several proteins known to be important for HIV-1 replication, as well as others discovered in high throughput si/shRNA screens. The block in shuttling imposed by HIV-1, for its own expression, provides further evidence of the magnitude of its control over host function.

DISCOVERY OF HIV-1 CAPSID DISASSEMBLY INHIBITORS

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To discover inhibitors of the HIV-1 replication cycle, 60,000 compounds were screened against replication incompetent HIV-1 pseudotyped with VSV-G, the envelope glycoprotein of vesicular stomatitis virus. A novel antiviral compound was identified from this screen (compound 1), exhibiting an EC_{50} of 7μM against wild type HIV-1 and a selectivity index (SI) greater than 10. The chemical synthesis of additional analogues of compound 1 established a structure activity relationship (SAR) for this series of antiviral inhibitors. These compounds act following reverse transcription and prior to nuclear import of the pre-integration complex (PIC), as determined by real-time PCR. Consistent with this observation, the activity of this series is maintained against non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant viruses. *In vitro* assembled capsid (CA) core-like structures are stabilized in the presence of these inhibitors, as measured by both CA assembly and disassembly assays. In agreement with their effect on CA stabilization, compound 1 binds to the N-terminal domain (NTD) of CA with a K_d of 16-20μM, as measured by isothermal calorimetry and NMR. An X-ray structure of compound 1 with the NTD of CA was obtained, demonstrating that Asn57 and Thr107 of the CA NTD pocket form three hydrogen bonds with the bicyclic portion of the molecule. Importantly, these amino acid residues are conserved in over 99% of HIV-1 isolate sequences deposited in the Los Alamos HIV sequence database. Mutations at either of these residues decrease the susceptibility of the virus to this series of compounds. In addition, drug resistant viruses with amino acid substitutions in the CA NTD pocket (A105T, G106E, T107A or T107N) emerged following the passaging of wild type HIV-1 in the presence of compound 2, a more potent analogue of compound 1 (EC_{50} of 1.4μM, SI>29). We propose that the inhibition of HIV-1 replication by these novel CA disassembly inhibitors involves the stabilization of CA cores, thereby blocking the nuclear import of the PIC and preventing further steps required for the propagation of the virus.

INHIBITION OF THE BUDDING/RELEASE OF PORCINE ENDOGENOUS RETROVIRUS

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Porcine endogenous retrovirus (PERV) is integrated in the genome of all pigs. Use of pigs for human xenotransplantation raises concerns about the risks of transfer of this infectious agent from the donor to xenotransplantation recipients. PERV consists of three replication-competent subtypes, PERV-A, PERV-B, and PERV–C. Whereas PERV-A and PERV-B are polytropic and can productively infect human cell lines, PERV-C is an ecotropic. Recombinant PERV-A/C is able to infect human cells and exhibits high titer replication. In this study, to establish the strategies to inhibit PERV production from the cells, we investigated the mechanism of PERV budding and the anti-PERV activity of Tetherin/BST-2.

First, we examined the cellular factors, which are involved in PERV budding, using the dominant-negative (DN) mutants of candidate cellular factors. The results showed that the DN mutants of Vps4A/B and Tsg101 markedly reduced PERV-A/C production in human and porcine cell lines. This suggests that PERV budding utilizes the cellular multivesicular body (MVB) sorting pathway as well as other many retroviruses. Moreover, PERV production was also reduced by expression of human and porcine Tetherin/BST-2. These data would be useful for development of strategies to inhibit PERV production and may reduce the risk for PERV infection in xenotransplantation.

M184V MUTATION INCREASES RNASE H CLEAVAGE-MEDIATED DISSOCIATION OF PRIMER-TEMPLATE BY HIV-1 REVERSE TRANSCRIPTASE (RT) CONTAINING AZT-RESISTANCE MUTATIONS.

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Replication of HIV-1 is inhibited by azidothymidine (AZT), which leads to chain termination and inhibition of DNA synthesis. Resistance to AZT is frequently the result of mutations that increase the ability of RT to remove the chain-terminating nucleotides after they have been incorporated. It has been proposed that RNase H cleavage of the RNA template can occur when RT is stalled near the site of chain termination and contributes to the inhibition by causing the dissociation of the primer-template before the chain terminator can be excised. Mutations in the connection and RNase H domains of RT have been shown to increase excision. It has long been known that resistance to thymidine analogs is conferred by the mutations M41L, D67N, K70R, L210W, T215F/Y and T219Q/E in RT and that this resistance is suppressed by the additional presence of the M184V mutation. Changes in excision activity on DNA templates have been observed with these mutant RTs, but effects on RNase H cleavage resulting in indirect effects on excision activity is also possible with RNA templates. We used 5'-labeled -3'-AZTMP-terminated DNA primer annealed to either DNA or RNA template to evaluate primer rescue activities, 5'-labeled RNA template to evaluate RNA cleavage activity, and biotin-tagged chain-terminated oligodeoxynucleotide to monitor primer-template dissociation.
Addition of M184V to thymidine analog resistance mutations decreased primer rescue to a much greater extent on RNA template than on DNA template, and M184V suppression of primer rescue was observed with either ATP or PPi as excision substrate. RNase H cleavage at secondary cleavage sites (-7, -8) was substantially reduced with M41L-T215Y RT in comparison with wild type RT, and primer-template dissociation was decreased. In contrast, when M184V was present, RNase H cleavage at the secondary cleavage sites and dissociation of the primer-template occurred at wild type levels or higher. M184V suppression of primer rescue was not observed when the RNase H-negative E478Q mutation was also present. These results suggest that RNase H-mediated RNA-DNA template-primer dissociation is influenced by mutations associated with thymidine analog resistance, and that suppression of resistance to nucleoside RT inhibitors by M184V may be partly explained by effects on RNase H cleavage that decrease the time available for excision to occur. These results suggest that mutations in the polymerase domain of RT can affect excision through an RNase H-dependent mechanism.

A SMALL MOLECULE INHIBITS HIV-1 INFECTION BY DESTABILIZING THE VIRAL CAPSID

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HIV-1 infection is dependent on proper disassembly of the viral capsid, or "uncoating," in target cells. The HIV-1 capsid consists of a conical polymer of the CA protein arranged in a hexagonal lattice. Mutations in CA that destabilize the viral capsid result in impaired infection owing to defects in reverse transcription in target cells. Here we describe the mechanism of action of a small-molecule HIV-1 inhibitor, PF-3450074 (PF74), that targets the viral capsid protein (CA). PF74 acts at an early stage of HIV-1 infection and inhibits reverse transcription in target cells. We show that PF74 binds specifically to HIV-1 particles, and substitutions in CA that confer resistance to the compound abolish drug association. A single point mutation in CA that stabilizes the HIV-1 core also confers strong resistance to the virus without inhibiting compound binding. Treatment of HIV-1 particles with PF74 resulted in destabilization of the viral capsid in vitro. PF74 antiviral activity was enhanced on binding of the host protein cyclophilin A to the HIV-1 capsid, and PF74 and cyclosporin A exhibited mutual antagonism. Finally, mutations in CA conferring resistance to PF74 stabilized the HIV-1 capsid and rendered the virus resistant to the host restriction factors TRIM5α and TRIMCyp. Our data suggest that PF74 induces premature HIV-1 uncoating in target cells, thereby acting as a small molecule mimetic of the restriction factor TRIM5α. The results highlight uncoating as a step in the HIV-1 life cycle susceptible to small molecule intervention.

UPF1 SHUTTLING FUNCTION IS REQUIRED FOR HIV-1 GENOMIC RNA EXPORT.

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The upframeshift supressor proteins (UPF1, 2 & 3) are core proteins of the major nonsense-mediated decay (NMD) surveillance machinery responsible for clearing mRNAs that contain premature termination codons (PTCs). UPF1 is also involved in RNA splicing, transport and translation. Previous studies from our laboratory identified UPF1 as a host protein involved in maintaining HIV-1 genomic RNA (vRNA) stability and translation. We also found UPF1 in the Gag ribonucleoprotein complex and in purified HIV-1 particles. We investigated the role of UPF1 in vRNA localization and the importance of the shuttling function of UPF1 in the fate of the HIV-1 vRNA. We found that UPF1 expression relocalized HIV-1 vRNA to the cytoplasm under Rev- conditions. Shuttling mutants of UPF1 lacking either the nuclear export (NES) or the nuclear localization signal (NLS) did not effect vRNA export under Rev- conditions. Moreover, UPF1 overexpression resulted in an increase in expression and the cytoplasmic relocalization of TAP/NXF1 with no observed change in CRM1. This suggests that this export pathway is favored by UPF1 for proper trafficking of the HIV-1 vRNA. Finally, the magnitude of the effect of UPF1 on HIV-1 vRNA levels and localization as well as those on Gag expression suggest that targeting UPF1 function might represent a suitable approach to block HIV-1 at the late expression phase.

FAILURE TO DETECT XMRV IN HUMAN PROSTATE TUMORS

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The presence of a gammaretrovirus, termed Xenotropic Murine leukemia virus Related Virus (XMRV), in human prostate tumors has been reported by several investigators. We have attempted to detect XMRV in prostate tumor samples using both real-time PCR on cellular DNA and immunohistochemistry (IHC) on tumor sections and tissue microarrays. Our PCR methods routinely detect the XMRV in 10 pg of DNA from 22Rv1 cells (an XMRV-infected prostate cancer cell line) or from cells infected in the laboratory. The admixture of 100-1000 ng of uninfected human DNA does not interfere with XMRV detection in these experiments. In the same PCR wells, a real-time assay for CCR5 DNA is simultaneously performed, in order to monitor the integrity of the cellular DNA.

To date, we have tested 146 prostate tumor DNA samples by PCR. Almost 1/3 of these assays used 100 ng or more of DNA, while 12 used DNA prepared from microdissected tumor tissue. Of the 95 samples for which less than 100 ng of DNA was used, 47 were tested by nested PCR.

In addition, we have tested tissue microarrays from over 400 prostate tumors by IHC, and full tissue sections from an additional 25 prostate tumors. Nearly all the tumor samples were from Gleason grade of 7 or above; over 50 of them were metastases. Our IHC uses broadly reactive anti-MLV CA and anti-MLV SU antisera; cells transfected with a molecular clone of XMRV show specific reactivity with both of these antisera.

In every case, the PCR and IHC results were indistinguishable from negative controls: thus we have not yet observed the presence of XMRV in prostate tumors. Our data raise the possibility that the prevalence of XMRV in prostate tumor patients may be much lower than originally reported.

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SELECTIVE DEGRADATION OF NEWLY SYNTHESIZED BST-2 DOES NOT EXPLAIN ENHANCED VIRUS RELEASE BY VPU

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Recent work identified BST-2/CD317/tetherin as a host factor whose expression results in the inhibition of HIV-1 virus release. It was suggested that BST-2 inhibits virus release by tethering viral particles to the cell surface and triggering their subsequent endocytosis and degradation. HIV-1 Vpu counteracts this effect but it is unclear how Vpu accomplishes this task.

Our initial analyses identified significant variation of endogenous BST-2 expression in HeLa cells, T cell lines, and primary cells that did not directly correlate to the degree of inhibition of particle release. While transient expression of codon-optimized Vpu led to efficient internalization of cell surface BST-2 in HeLa cells we failed to observe similar cell-surface down-modulation of endogenous BST-2 during acute infection of T cell lines or primary cells. We concluded that cell surface downregulation of BST-2 and augmentation of virus release from BST-2 expressing cells are separable properties of Vpu.

In an extension of this study we performed kinetic studies to determine how Vpu affected cell surface downregulation of BST-2. Vpu must either affect the turnover of BST-2, internalization rate of BST-2 from the cell surface, or the recycling rate of BST-2. We agree with previous reports that Vpu does not affect the internalization rate of BST-2 from the cell surface. Instead, we found that Vpu increases the turnover of newly synthesized BST-2 in transfected 293T cells in a TrCP-dependent manner. Importantly, however, Vpu did not increase the turnover of mature BST-2. Results will be presented addressing the differences in Vpu sensitivity between transiently expressed newly synthesized BST-2 and pre-existing endogenous BST-2. The rate of de novo synthesis of endogenous BST-2 is very low when compared to transiently expressed protein and we predict that depletion of endogenous BST-2 by inhibiting de novo synthesis may be too slow to significantly impact virus release. We predict that while Vpu might have a slight effect on the turnover of BST-2 this could not result in the degree of inhibition observed. Therefore BST-2 recycling is likely to be affected by Vpu through a degradation-independent mechanism.

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE PROTEINS TRAFFIC TOWARD VIRION ASSEMBLY SITES VIA A TBC1D20/RAB1/2-REGULATED PATHWAY

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The interaction of the human immunodeficiency virus type 1 (HIV-1) with the cell is complex. To uncover cellular factors that influence HIV-1 replication several systematic siRNA screens have recently been preformed; each listed hundreds of such factors, with minimal overlap between the different lists. Thus, evaluation of the relevance of these specific factors to HIV-1 replication is still needed. In these screens, the small GTPases Rab1 and 2, known to regulate anterograde traffic between the endoplasmic reticulum (ER) and the Golgi complex, were identified; Rab1 depletion reduced HIV-1 infectivity and this factor was suggested to act in late stages of infection. To evaluate Rab1/2-HIV-1 interactions, we over-expressed TBC1D20, the recently discovered Rab1/2 GTPase-activating protein (GAP), since TBC1D20 overproduction has been shown to block Rab1/2-mediated ER-to-Golgi transport. We found that over-expression of TBC1D20, but not its catalytic-inactive form, together with HIV-1 Gag and envelope (Env) proteins, resulted in abnormal gp41 migration in SDS-PAGE indicating atypical cellular modification of the Env protein, likely because of aberrant glycosylation. Moreover, TBC1D20 over-expression did not reduce virion like particles (VLPs) production but decreased particle infectivity and Env incorporation into HIV-1 VLPs. Immunofluorescence analysis revealed ER retention of Env in the TBC1D20-expressing cells. Taken together, these results demonstrate that HIV-1 Env is processed and migrate towards the virion assembly sites via a TBC1D20/Rab1/2-dependent secretory pathway.

HIV-1 SUBTYPE B AND C INTEGRASE ENZYMES EXHIBIT DIFFERENTIAL PATTERNS OF RESISTANCE TO INTEGRASE INHIBITORS IN VITRO

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Background: Integrase inhibitors are now a component of highly active antiretroviral therapy (HAART) regimens, having now been approved for both first-line and salvage therapy. Due to the high inter-subtype genetic variability of HIV-1, the potential for subtype-specific patterns of resistance to integrase inhibitors exists.
Methods: This study compares the susceptibility of HIV-1 integrase enzymes harbouring previously reported resistance mutations E92Q, N155H and E92Q+N155H, from both subtypes B and C to clinically relevant integrase inhibitors. This was performed biochemically using a microtiter plate system.
Results: Our results show the existence of subtype-specific differences in the susceptibility of integrase enzymes to integrase strand transfer inhibitors. Subtype C integrase bearing resistance mutations E92Q+N155H derived from the molecular clone pINDIE-C1 was approximately 10-fold more susceptible to both Raltegravir and Elvitegravir in vitro, when compared to subtype B integrase derived from the molecular clone pNL4-3.
Conclusions: Polymorphic differences between subtype B and C likely cause variations in the contribution of the N155H, alone or in combination with E92Q mutation, to integrase inhibitor resistance. This suggests different subtypes might favour different mutational pathways, which could lead to viral subtypes exhibiting different levels of resistance to integrase inhibitors.

FUNCTIONAL COMPARATIVE ANALYSES OF GLYCOSYLATED GAG MOLECULES DERIVED FROM DIFFERENT GAMMARETROVIRUSES

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Glycosylated Gag is a type II MLV transmembrane protein translated from an initiation CUG codon upstream and in-frame with the ordinary Gag. We have recently reported that glycosylated Gag is an infectivity factor which can replace the activity of Nef on HIV-1 infectivity and shares with Nef major functional features. A potential ORF encoding glycosylated Gag can be found in the genome of many exogenous and endogenous gammaretroviruses. However, there is yet no evidence that all these viruses are capable of producing the predicted protein.
We wanted to assess the ability of several retroviruses to encode glycosylated Gag and test whether the activity of these molecules on retrovirus infectivity is a conserved property. To this end we have analyzed retrovirus genomes and selected individual viruses from different gammaretrovirus species which contain an ORF encoding a potential glycosylated Gag. All protein sequences subjected to in silico analyses were predicted to have a type II transmembrane topology. However, an initiation codon for glycogag could not be found within the sequences derived from gibbon ape leukemia virus.
We have cloned the 5' portion of retroviral genomes and the putative glycosylated Gag ORF from genomic RNA derived from several gammaretroviruses. In contrast to the known genome sequences, a conserved glycogag CUG initiation codon is present in genomes of both SEATO and X strains of GALV. The predicted ORF encode a protein with a cytoplasmic tail highly divergent from that of glycosylated Gag derived from MLV. We are currently exploring the activity of all glycogag alleles on gammaretrovirus and lentivrus infectivity. This will elucidate the aminoacid sequence determinants and the common features functionally important for the Nef-like function of glycosylated gag on retrovirus infecitivity.

MECHANISMS OF TYPE-I INTERFERON TRANSCRIPTIONAL REGULATION OF TETHERIN, A HOST FACTOR RESTRICTING RETROVIRUS RELEASE.

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Tetherin is an interferon (IFN)-regulated host restriction factor whose expression blocks the release of HIV-1 and other enveloped viral particles. While the molecular underpinning of Tetherin potent inhibition of HIV-1 release has been well defined, the endogenous regulation of this antiviral effector has not been fully elucidated. Using flow cytometry, we first established that Tetherin is expressed under basal conditions in a number of cell types, including ex-vivo primary human T lymphocytes and cells of myeloid linage such as monocytes and macrophages. While Tetherin can be mildly induced upon cell culture, its expression is greatly stimulated by T cell activation by either PHA or anti-CD3/anti-CD28 stimuli. Furthermore, as previously reported, we found that surface expression of Tetherin could be further induced following exposure to type-I IFN on all the cell types tested.

Using a dual luciferase reporter assay and model cell lines that either constitutively express Tetherin (HeLa) or express Tetherin only after IFN induction (HEK293T), we identified a minimal promoter region required for Tetherin basal and IFN-inducible expression. Tetherin minimal promoter consists of a 100base pair region containing multiple overlapping transcription factor binding sites, including canonical type-I and type-II IFN response elements (ISRE and GAS sites respectively), NF-AT, IRF-1 and STAT-3 binding sites; as well as two putative non-traditional tata boxes. Mutations in a 16bp segment, containing one ISRE and two IRF1 binding sites, completely abolished promoter capacity for type-I IFN induction without, however, affecting its basal expression. Interestingly, IFN-mediated induction could be rescued by reconstituting only one IRF-1 binding site, suggesting a potential role of IRF-1, a key product of type-I IFN signaling cascade, in the induction of Tetherin expression by IFN. In that regard, we demonstrated that over-expression of IRF-1 in HEK293T indeed induced the expression of Tetherin. Finally, we investigated the role of STAT-1, an early component of type-I IFN signaling cascade, in Tetherin expression. We found that induction of Tetherin expression by type-I IFN required STAT-1 phosphorylation, whereas Tetherin basal expression was independent from STAT-1 protein. Using specific inhibitors and shRNA-directed depletion we are currently analyzing further the interplay between STAT-1 and IRF-1 on Tetherin transcriptional activation by type-I IFN.

Overall, our results indicate that a complex set of IFN-inducible proteins is involved in Tetherin transcriptional regulation. Insights into the molecular pathways regulating Tetherin expression may provide key information for the development of rational strategies aimed at manipulating levels of endogeneous Tetherin for enhancing antiviral immunity.

BIOCHEMICAL SEPARATION OF HIV-1 REVERSE TRANSCRIPTION AND PREINTEGRATION COMPLEXES

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The early steps of HIV-1 replication are characterized by two operationally defined nucleoprotein complexes- the reverse transcription complex (RTC) and the preintegration complex (PIC). The RTC facilitates reverse transcription of the viral RNA into DNA, and the PIC mediates integration of the viral DNA into the host chromosome. The architecture and temporal regulation of these complexes are unclear, and it remains controversial whether these complexes are biochemically distinct. The biochemical study of intact complexes has been hampered by both their limited quantity in cells and their labile nature. Previous studies have purified RTCs and PICs by both density/equilibrium and velocity centrifugation, and size-exclusion chromatography. We report in this study that these complexes can be biochemically separated using velocity gradient (rate-zonal) centrifugation and fractionation. Distinct viral RNA/reverse transcriptase active and viral DNA/integration active complexes were separable using short duration 5-45% sucrose gradients. The sedimentation of the RTC was slower than the PIC suggesting it is smaller in size. Both complexes behaved differently under varying centrifugal force. The RTC displayed constant sedimentation for up to 4 h. In contrast, the PIC rapidly disintegrated- by 4 h there was a loss of recoverable integration activity, and a dissociation of the majority of vDNA from fractions containing the integration activity. If equilibrium gradients were used, both the RTC and PIC factors concentrated to same fractions of the gradient. These results demonstrate the biophysical properties of RTCs and PICs in sucrose gradients, illustrate the labile nature of PICs under high speed centrifugation, and define a protocol to biochemically separate RTCs and PICs.

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CELL TYPE SPECIFIC ANTIVIRAL EFFECTS OF APOBEC3A DURING THE EARLY PHASES OF LENTIVIRAL INFECTION OF MYELOID CELLS AND POSSIBLE COUNTERACTING VIRAL MEASURES

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The early phases of lentiviral infection are generally completed less efficiently in cells belonging to the myeloid lineage than in other cell types and this behavior is largely influenced by their differentiation status and the exogenous stimuli present. The reasons for this more restrictive phenotype are largely unknown and have been ascribed either to a general low metabolic state or to the cell type specific expression of cellular factors with antiviral activity. To date only Vpx, a viral protein coded by members of the HIV-2/SIVSM lineage, seems to be able to overcome the restrictive phenotype of myeloid cells and to allow their efficient infection by a wide panel of lentiviruses.
Among known antiviral factors, human APOBEC3A (hA3A) retained our attention, as this protein seems expressed almost exclusively in myeloid cells, variations in its expression seem to correlate with differences in the cells' susceptibility to infection and a previous study indicated that downregulation of this factor in non-stimulated monocytes led to increased replication of HIV-1. In contrast, overexpression of hA3A in non-myeloid cells seems to bear no consequences on the virus, suggesting a possible cell type specific effect. Whether hA3A acts directly on incoming viruses during the early phases of infection or else, like other members of the APOBEC family, if it acts during assembly to negatively imprint virions remains unknown.
To gather novel insights into the role that hA3A may exert during viral infection, we have first examined the kinetics of its expression during ongoing viral replication in primary macrophages and showed that hA3A upregulation occurs naturally during infection with both HIV-1 and HIV-2, when expression of other members of the APOBEC family is unchanged. Then, we have examined the role of hA3A specifically during the early phases of the viral life cycle. Knock down experiments indicate that hA3A exerts an antiviral activity that targets similarly HIV-1, HIV-2 and SIVMAC. The characterization of this antiviral effect, as well as the possible relationship between hA3A and the viral HIV-2/SIVSM Vpx protein will be presented here.

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THE ESCRT-0 COMPONENT HRS IS REQUIRED FOR HIV-1 VPU-MEDIATED BST2/TETHERIN DOWN-REGULATION.

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The ESCRT machinery, a highly conserved set of four hetero-oligomeric protein complexes, is required for multivesicular body formation, sorting ubiquitinylated membrane proteins for lysosomal degradation, cytokinesis and the final stages of assembly of a number of enveloped viruses, including the human immunodeficiency viruses. Here, we show an additional role for the ESCRT machinery in HIV-1 release. BST2/tetherin is a restriction factor that impedes HIV release by tethering mature virus particles to the plasma membrane. We found that HRS, a key component of the ESCRT-0 complex, promotes efficient release of HIV-1 and that siRNA-mediated HRS depletion induces a BST2/tetherin phenotype. This activity is related to the ability of the HIV-1 Vpu protein to down-regulate BST2/tetherin. We found that BST2/tetherin undergoes constitutive ESCRT-dependent sorting for lysosomal degradation and that this degradation is enhanced by Vpu expression. We demonstrate that Vpu-mediated BST2/tetherin down-modulation and degradation require HRS (ESCRT-0) function and that knock down of HRS increases cellular levels of BST2 and restricts virus release. Our results provide further insight to the mechanism by which Vpu counteracts BST2/tetherin to favour HIV-1 dissemination and highlight an additional role for the ESCRT machinery in virus release.
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MUTAGENIC SCREEN-BASED SELECTION OF TRIM5A$_{HU}$ MUTANTS THAT POTENTLY RESTRICT HIV-1

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The restriction factors TRIM5α and the related TRIMCyp are potentially excellent gene candidates for AIDS therapeutic strategies involving gene transfer. TRIM5α can neutralize incoming HIV-1 very early in its replication cycle, before any viral protein is being synthesized de novo. Rhesus macaque TRIM5α (TRIM5α_{rh}) strongly restricts HIV-1 while human TRIM5α is inactive against this virus but can inhibit other retroviruses. An attractive alternative to using potentially immunogenic nonhuman primate versions of TRIM5α is the generation of human TRIM5α variants bearing a small number of mutations leading to efficient inhibition of HIV-1.

So far, identifying the determinants of HIV-1 targeting by TRIM5α has been done mostly by searching for differences between the PRYSPRY domain sequence of TRIM5α_{hu} and those of nonhuman primates. My lab has undergone two mutagenic screens to generate novel variants of TRIM5α_{hu} with strong HIV-1 restriction activity. One approach involves error-prone PCR of the whole PRYSPRY domain while the other one uses a degenerated oligonucleotide and is limited to the first variable loop of PRYSPRY. This strategy has allowed us to isolate two clones in which resistance was due to expression of TRIM5α_{hu} mutated at positions 335 (R335G) or 330 (G330E), both located in the v1 loop. Mutations at arginine 332, previously shown by others to be an important determinant of HIV-1 restriction, were also found. Individually, each mutation resulted in a 10-fold or more decrease in human cells permissiveness to transduction by HIV-1 vectors. Mutations found at G330, R332 and R335 all affected the charge of the v1 loop, and targeted mutagenesis at positions 330 and 335 showed that increased acidity was indeed required for HIV-1 restriction but was not sufficient. R332G and R335G had partly additive effects while mutations at G330 did not enhance HIV-1 restriction by TRIM5α_{hu} mutated at positions 332 or 335. The double mutants R332G/R335G and R332G/R335E inhibited HIV-1 spreading infection in the T cell line SupT1 as efficiently as TRIM5α_{rh} did, confirming their potential as anti-retroviral transgenes.

We are currently characterizing the mechanism of HIV-1 restriction by these mutant TRIM5α_{hu} and monitoring the possible appearance of HIV-1 resistance. Our recent data indicate that in human cells, pharmacological inhibition of cyclophilin A by cyclosporine A decreases the restriction of HIV-1 by the various mutant TRIM5α_{hu} generated in our screens. Therefore, cyclophilin A modulation of HIV-1 restriction by Old World monkeys TRIM5α is not a peculiarity of these TRIM5α orthologues but seems to be a general phenomenon.

CHARACTERIZATION OF CELLULAR DETERMINANTS REQUIRED FOR INFECTION OF XMRV, A NOVEL RETROVIRUS ASSOCIATED WITH HUMAN FAMILIAL PROSTATE CANCER

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The newly identified retrovirus – the xenotropic MuLV-related virus XMRV – has recently been shown to be strongly associated with familial prostate cancer in human (Urisman et al., 2006, PLos Pathog). While that study showed evidence of XMRV infection exclusively in the prostatic stromal fibroblasts, a recent study found XMRV protein antigens mainly in malignant prostate epithelial cells (Schlaberg et al., 2009, PNAS). To help understand the mechanisms behind XMRV infection, we show that prostatic fibroblast cells express Xpr1, a known receptor of XMRV, but its expression is absent in other cells of the prostate (i.e., epithelial and stromal smooth muscle cells). We also show for the first time that certain amino acid residues located within the predicted extracellular loop (ECL3 and ECL4) sequences of Xpr1 are required for efficient XMRV entry. While we found strong evidence to support XMRV infection of prostatic fibroblasts via Xpr1, we learned that XMRV was indeed capable of infecting cells that did not necessarily express Xpr1, such as those of the prostatic epithelial and smooth muscle origins. Further studies suggest that the expression of Xpr1 and certain genotypes of the RNASEL gene, which could restrict XMRV infection, may play important roles in defining XMRV tropisms in certain cell types. Collectively, our data reveal important cellular determinants required for XMRV infection of human prostate cells in vitro, which may provide important insights into the possible role of XMRV as an etiologic agent in human prostate cancer.

VIF PROTEINS OF VARIOUS HIV-1 SUBTYPES EXHIBIT DIFFERENT ACTIVITY AGAINST APOBEC3G.

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APOBEC3G (A3G) restricts HIV-1 replication by editing and non-editing mechanisms. The Viral Infectivity Factor (Vif) protein counteracts APOBEC3G action by preventing its incorporation into HIV-1 virus particles. Previous studies have shown variation in the anti-A3G activity of naturally-occurring subtype B Vif proteins.

Because of the pronounced sequence diversity between HIV-1 subtypes, we hypothesized that Vif proteins of different subtypes vary in their anti-A3G activity. We assessed 17 Vif proteins of seven subtypes (A, B, C, D, E, F and G) for their ability to degrade A3G as well as to rescue HIV-1 infectivity in the presence of A3G in single-cycle infections. HIV-1 NL4-3 viruses were generated by co-transfection of the different Vif proteins with the NL4-3ΔVif molecular clone. Expression of tagged and untagged Vifs was assessed by western blot.

All HIV-1 Vifs from the tested panel were active against A3G with the exception of a subtype E Vif protein. However the anti-A3G efficacy varied significantly, with a 7-fold difference between the most and least active of the Vif proteins. Such diversity in activity occurred both within subtypes as well as between subtypes. Interestingly, some non-subtype B Vif proteins performed better than the Vif proteins from the HIV-1 subtype B reference clones NL4-3 and LAI.

Our work highlights the importance of assessing 'Vif-A3G' phenotypes since Vif proteins from different HIV-1 subtypes vastly differ in their efficiency to counteract A3G. The differences in Vif activity observed in single-cycle infection experiments are likely to be amplified in the context of a spreading infection. More HIV-1 Vif genotype to phenotype determinations are needed since differences in Vif function may impact HIV-1 transmission, replication and pathogenesis.

HIV INCORPORATION OF A HOST DERIVED GANGLIOSIDE IS CRUCIAL IN MEDIATING DENDRITIC CELL CAPTURE AND TRANS INFECTION TO $CD4^+$ T CELLS.

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The interaction between human immunodeficiency virus (HIV) and dendritic cells (DCs) is an important early event in HIV pathogenesis that leads to efficient viral dissemination. The majority of virus that is captured by DCs remains in an infectious form and is passed to $CD4^+$ T cells through a process of *trans* infection. All of the DC specific attachment factors that have been described to date rely upon interactions with the highly variable HIV glycoprotein gp160. Our lab has recently demonstrated that DC capture and transfer of HIV can be glycoprotein independent, using instead a host derived glycosphingolipid (GSL) that is incorporated into the virus as it buds from the lipid raft of the producer cell. Using eGFP containing virus-like particles (VLPs), we show that this GSL dependent DC binding mechanism extends to the paramyxoviruses Hendra and Nipah, which also bud from GSL enriched lipid rafts and compete HIV Gag-eGFP VLPs for binding to DCs. In all cases, viruses produced in the presence of the GSL biosynthesis inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) show an approximate eighty percent decrease in DC capture. Decreased levels of DC capture translate directly into a decreased efficiency in establishing trans infection, suggesting this interaction plays a key role in transit of the virion through the DC. Similarly to PDMP inhibition, knocking down GSLs in the producer cell using RNAi directed against the glucosyltransferase resulted in inhibition of HIV capture by DCs. More specifically, the knockdown of ganglioside GM3 synthase enzyme but not GM2 synthase or globotriaosylceramide synthase within the producer cell also resulted in the inhibition of HIV capture by DCs. Finally, addition of GM3 to GSL-depleted virus producer cells to selectively enrich GM3 within HIV-1 particles resulted in a substantial enhancement of DC capture as compared to the capture of GSL deficient virus. This suggests that of the larger class of GSLs, the ganglioside GM3 is mediating the interaction between HIV and DCs. Since GM3 is a host cell-derived molecule that is also enriched within exosomes, this interaction may help the virus to successfully navigate past the degradation pathway in DCs. HIV binding via GM3 describes an important interaction between the virus and target cell that is devoid of the high mutation rate inherent in virally encoded proteins, while also suggesting a novel mechanism by which HIV evades degradation within the DC and ultimately establishes productive *trans* infection to the $CD4^+$ T cell.

A HOST RESTRICTION ACTING AGAINST MMTV AND HBRV GAG GENE EXPRESSION REVEALS A NEW LEVEL OF HOST REGULATION OF RETROVIRAL GENE EXPRESSION

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Human betaretrovirus (HBRV) is >90% homologous to mouse mammary tumor virus (MMTV), and its isolation from patients with primary biliary cirrhosis could represent a zoonosis. In experiments to test the possibility that HBRV Gag expression could recapitulate the cell surface expression of mitochondrial antigens associated with the pathology of PBC, we were unable to achieve CMV-driven HBRV Gag protein expression. The endogenous MMTV (MT) *gag* gene derived from an infectious molecular clone also failed to express under identical conditions, but MT Gag was readily detectable when expressed from an intact provirus. HBRV and MT Gags were equivalently expressed in rabbit reticulocyte lysates, leading us to hypothesize that human cells restrict MMTV and HBRV Gag expression and that the intact provirus can overcome this restriction. Here we describe studies to identify the mechanism of this novel restriction/antirestriction nexus.

An exogenous MMTV *gag*, (SM), silently mutated in pp21 to support bacterial propagation, expresses to high levels in human cells. Chimeras between the restricted HBRV or MT and the non-restricted SM *gag* genes allowed the identification of the target of restriction as a 54 nt sequence in the code for the pp21 domain.

To identify the step at which restriction occurs we quantified transcript abundance, nuclear to cytoplasmic mRNA transport, and mRNA stability among the constructs. Each was found to be equivalent. Proteasome inhibition and metabolic labeling demonstrated that lack of detectable HBRV/MT Gag protein was not due to protein instability, but rather to a pre-translational block. Consistently, we found that HBRV *gag* blocked expression of a downstream IRES GFP cassette in a bicistronic vector, whereas the SM *gag* did not, implying that the restriction acts on the message itself. These results were consistent with the concept that restriction was due to a microRNA. However, Gag expression remained restricted in cells where Dicer was knocked down using siRNAs. In agreement with these findings, when mRNA for either restricted or non-restricted *gags* was introduced into cells no restriction was evident. Our results suggest that the HBRV/MT mRNA is negatively regulated for translational competence due to exposure to the human nucleus. We are now investigating whether nuclear factors prevent the restricted messages from interacting with the ribosomes or target them to regions in the cytoplasm where they are inaccessible for translation. Our findings reveal a novel layer of complexity in the regulation and restriction of retroviral lifecycles in human cells.

IDENTIFICATION OF NOVEL ASSEMBLY DOMAIN AT THE N-TERMINUS OF MASON-PFIZER MONKEY VIRUS NUCLEOCAPSID PROTEIN

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Assembly of immature retroviral particles is a complex process mediated by mutual interactions of Gag polyproteins. In all retroviruses, the Gag precursor contains matrix (MA), capsid (CA) and nucleocapsid (NC) protein and the latter two represent the key interaction domains. Bacterial expression/assembly system, *in vitro* and *in vivo* assembly assays were used to study the effect of N-terminal domain of NC on formation of Mason-Pfizer monkey virus (M-PMV) particles. Analyzing of truncated and mutated constructs of CANC fusion proteins or whole Gag we identified a "spacer-like" region consisting of fifteen N-terminal amino acids of the NC (NC15). This M-PMV "spacer-like" peptide is indispensable for correct assembly and cannot be replaced with dimerizing or trimerizing domains or with the HIV-1 spacer peptide sequence. However, this NC15 sequence is not sufficient to mediate the assembly when fused to CA. To induce the assembly of virus-like particles, the CANC15 must be followed by a short sequence that is rich in basic residues. This basic region can be either specific, i.e. derived from the downstream NC sequence, or it can be substituted by non-specific positively charged peptide. Efficient assembly of M-PMV immature-virus-like particles was observed only in the presence of both "spacer-like" sequence and basic region.

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NMR STRUCTURAL STUDIES AND MOLECULAR MODELING OF THE INTERACTION BETWEEN THE SPACER PEPTIDE SP1 AND BETULINIC ACID DERIVATIVES

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Assembly and maturation of HIV-1 Gag precursor constitute a target for the design of new molecules able to inhibit virus replication. The polyprotein Gag is maturated by the viral protease (PR) to give matrix (MA), capsid (CA), nucleocapsid (p7), p6 and two extra spacer peptides (Sp1 and Sp2), separating CA/p7 and p7/p6 respectively. The peptide Sp1 plays a crucial role in the assembly and in the maturation of HIV-1. Mutations within Sp1 or at the CASp1 junction perturb these steps that result in the production of virions with aberrant cores. In the same way, the inhibition of the CASp1 cleavage causes the production of defective and noninfectious viral particles. Betulinic acid derivatives (DSB / PA-457 / Bevirimat) are potent maturation inhibitors against HIV-1 replication by interfering with PR at the CASp1 cleavage site. DSB has also an effect on the assembly of HIV-1 Gag precursor as shown in vivo, into membrane-enveloped virus-like particles (VLP) in baculovirus-infected cells (Sf9) expressing Pr55GagHIV.

To attempt to elucidate the role of Sp1 and to determine the mechanism of action of the DSB during assembly or maturation of the virion, structural studies by NMR and Molecular Modeling have been initiated. The structures of the junction CASp1p7 as well as the mutated junction CASp1p7(A1V) have been determined by NMR. The main structural motif is a well-defined amphipathic _-helix that seems to be bent in the case of the mutated domain. The interaction between DSB and the CASp1p7 junctions has been undertaken by NMR to propose a structure of the complex and a mechanism for the inhibitor. NMR study of both partners was difficult due to the lack of solubility of DSB in water as well as in presence of organic solvents. For this reason, new DSB derivatives, with higher solubility and containing various C-28 amide modifications, were synthesized, characterized by NMR and evaluated for their anti-HIV activity by the measure of their IC50. Results show that the activity of DSB derivatives could be related to their solubility and that increasing their solubility could make possible their structural studies by NMR in association with Gag fragments. The structure of such complex should help to elucidate the mechanism of inhibition of DSB in assembly and maturation of HIV-1.

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LLAMA SINGLE-DOMAIN ANTIBODY FRAGMENT FOR INHIBITION OF THE NEF REGULATORY PROTEIN OF HIV-1

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The Nef regulatory protein of HIV-1 is essential in vivo for virus replication and AIDS pathogenesis. Despite this critical role in AIDS, Nef is not targeted by current antivral strategies. Nef exerts a positive influence on virus infectivity and induces profound functional perturbations of host cell endocytic and signaling pathways. Here, we describe the generation as well as biochemical and virological characterization of a camelid single-domain antibody (sdAb) that specifically binds to HIV-1 Nef with a high affinity (Kd = 2x10-9 M). When expressed as an intracellular antibody ("intrabody") in Nef-expressing or HIV-1-infected cells, this anti-Nef sdAb inhibited the critical biological activities of Nef. First, it interfered with the CD4-downregulation activity of Nef through inhibition of the Nef effects on CD4 internalization from the cell surface. Second, it was able to interfere with the association of Nef with activity of the cellular kinase PAK2 as well as with the resulting inhibitory effect of Nef on actin remodeling. Third, anti-Nef sdAb counteracted the Nef-dependent enhancement of virion infectivity. Since all these Nef functions have been implicated in Nef action in vivo, this anti-Nef sdAb represents an efficient tool to elucidate the molecular functions of Nef in the virus life cycle and will guide the development of new strategies for the suppression of AIDS pathogenesis.

INHIBITION OF CDK2 AFFECT CDK9 ACTIVITY AND PHOSPHORYLATION AND INHIBITS HIV-1

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Transcription of HIV-1 viral genes is activated by viral protein Tat and also uniquely dependent on the interaction of the viral HIV-1 Tat protein with host cell factors, including protein kinases CDK9 and CDK2. CDK9/Cyclin T1 (P-TEFb) is recruited to HIV-1 transcripts through the interaction with Tat protein and the TAR element located in HIV-1 transcripts. Our research focuses on the function of CDK2 in the regulation of HIV-1 transcription. HIV-1 replication is inhibited in the cells in which CDK2 expression is inhibited through stable expression of CDK2-targeting shRNA (293T-59 cells). Interestingly, inhibition of CDK2 also inhibited CDK9 activity. We analyzed whether CDK2 can phosphorylate CDK9 and found that siRNA-mediated inhibition of CDK2 reduced phosphorylation of CDK9 in vivo. Using CDK9-derived synthetic peptides containing potential phosphorylation sites for CDK2, we showed that Ser90 is likely the phosphorylation site. To further analyze the effect of CDK2 on CDK9 we analyzed distribution of CDK9/cyclin T1 between small active and large inactive complex in the cells in which CDK2 expression was inhibited by CDK2-directed shRNA. In the large complex P-TEFb is bound by 7SK RNA and hexamethylene bisacetamide-inducible protein 1 (HEXIM1) and Tat recruits P-TEFb from this complex. Using ultracentrifugation in glycerol gradients and differential salt extraction, we found less CDK9 and cyclin T1 in the large complex of the cells that lack CDK2. Our observations suggest that inhibition of HIV-1 in the absence of CDK2 may be due to the disappearance of the large CDK9/cyclin T1 complex. Taken together, our studies show that CDK2 can potentially phosphorylate CDK9 and that inhibition of CDK2 inhibited CDK9 activity and also inhibited HIV-1 transcription. Our results point to CDK2 as a good candidate for HIV-1 drug therapy.

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BOTH HUMAN AND RHESUS MACAQUE TRIM5A CAN BIND HIV-1 CORES AND STABILIZE CA DISASSEMBLY

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After membrane fusion with a target cell, the core of human immunodeficiency virus type 1 (HIV-1) enters into the cytoplasm where uncoating occurs. The cone-shaped core is composed of a hexameric lattice of the viral capsid protein (CA), which disassembles during uncoating. The underlying factors and mechanisms governing uncoating are poorly understood. Cellular proteins have been implicated in regulating uncoating, in both positive and negative manners. TRIM5α is a host restriction factor that targets CA and accelerates uncoating. This restriction occurs in a species-specific manner, whereby HIV-1 replication is weakly restricted by human TRIM5α (TRIM5α-Hu), but potently restricted by rhesus macaque TRIM5α (TRIM5α-Rh). The TRIM5α restriction requires CA recognition but the underlying mechanism is not completely understood. The weak restriction for HIV-1 by TRIM5α-Hu is presumably due to an inability to recognize the incoming viral core, based on binding assays with *in vitro* assembled CA-NC complexes, compared to strong affinity with TRIM5α-Rh. Using isolated cores in binding assays, both TRIM5α-Hu and TRIM5α-Rh were able to recognize and co-sediment with cores through a sucrose cushion, confirming that CA binding is necessary but not sufficient for restriction. By using an *in vitro* uncoating assay, which measures the rate of CA disassembly at 37°C over time, CA disassembly was not accelerated, but stabilized in the presence of TRIM5α-Hu or TRIM5α-Rh. The results show that both TRIM5α-Hu and TRIM5α-Rh are able to recognize HIV-1 viral cores and lead to the generation of a stable viral core-TRIM5α complex, which may represent an intermediate in the TRIM5α restriction process. The results indicate that TRIM5α-mediated inhibition of viral infection not only requires CA-binding, but is consistent with a model in which the restriction mechanism may occur in a step-wise manner and is dependent on additional and yet unidentified cellular factors.

ISOLATION OF NEWLY INTEGRATED DNA USING MU TRANSPOSITION IN VITRO

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We describe improved methods for isolating integration site sequences from infected cells, and their use in quantitative analysis to track transduced clones. Human genetic diseases have been successfully corrected by integration of functional copies of the defective genes into the human genome, but in some cases integration of therapeutic vectors has activated proto-oncogenes and contributed to leukemia. For this reason, extensive efforts have focused on analyzing integration site populations from patient samples, but previously used methods for recovering newly integrated DNA have been labor-intensive and suffered from severe recovery biases. Here we show that a new method based on phage Mu transposition in vitro allows convenient and consistent recovery of integration site sequences in a form that can be analyzed directly using pyrosequencing. Recovery is much less biased than methods requiring cleavage of the human genome with restriction enzymes, which are strongly influenced by the proximity of integration sites to restriction sites. The method also allows simple estimation of the relative abundance of gene-modified cells from human gene therapy subjects, which has previously been lacking but is crucial for detecting expansion of cell clones that may be a prelude to adverse events.

ROLE OF RAB7 IN THE PRODUCTION OF HIV-1 PARTICLES

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It's becoming increasingly clear that retroviruses take advantage of cellular trafficking machineries to assemble and release new HIV particles. Several studies have highlighted the crucial role of ESCRT machinery in the late stage of HIV assembly, but also the importance of the clathrin adaptor protein complexes, the Golgi-localized γ-ear containing Arf-binding and ADP ribosylation factor proteins, in HIV release. It was extensively described that Rab proteins regulate specific steps of intracellular membrane trafficking, including exocytosis and endocytosis in eukaryotic cells, by the recruitment of tethering, docking and fusion factors as well as the actin- and microtubule-based motor proteins to facilitate vesicle traffic. In order to identify new intracellular pathway required for HIV morphogenesis, we explored the role of 8 ubiquitously expressed Rab proteins (Rab1a, Rab4a, Rab5a, Rab6a, Rab7a, Rab8a, Rab9a, Rab11a) involved in the main endocytic and exocytic pathways, on HIV replication cycle.
For this purpose, we developed several virological tests based on the specific interference RNA targeting Rab proteins (Rab1, Rab4, Rab5, Rab6, Rab7, Rab8, Rab9 and Rab11). Here we show that Rab7 plays a major role on HIV-1 replication. We observed that HIV-1 (NL4-3 strain) fails to propagate in the reporter cells HeLa P4R5 (stably expressing CD4 and CCR5) upon siRNA–induced depletion of Rab7. Analysis of the early step of HIV replication cycle showed no major effect of Rab7 silencing on HIV entry. By contrast, study of the late steps of the HIV replication cycle using a single cycle infection assay showed that Rab7 depletion strongly increases the level of cell-associated processed Gag products, CAp24 and MAp17, and reduces the release of CAp24 from HeLa producer cells. Furthermore, we noted that this diminution of the viral release is associated with a modification of Gag processing and with a marked decrease of the infectious titer of the produced particles. Interestingly, no accumulation of cell-associated processed Gag was detected in Rab7-depleted HEK 293T cells culture products, whereas the infectious titer of produced particles remained lower in this culture compared to the control. Altogether, our current results reveal that Rab7 participates to the efficient production of full-infectious viral particles by acting at different stages during the late steps of HIV replication cycle.

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IDENTIFICATION OF SPECIFIC RESIDUES WITHIN THE L2 REGION OF RHESUS TRIM5A THAT ARE REQUIRED FOR RETROVIRAL RESTRICTION AND CYTOPLASMIC BODY LOCALIZATION

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The TRIM5α protein is an intracellular restriction factor known to inhibit infection by numerous retroviruses in a species specific manner. The most well studied example of this restriction is the TRIM5α protein from rhesus macaques (rhTRIM5α), which potently inhibits HIV-1 infection. TRIM5α is observed to localize to cytoplasmic accumulations of protein referred to as cytoplasmic bodies, though the role that these bodies play in the restriction of retroviruses remains unclear. We employed a series of rhTRIM5α truncation mutants to identify a discrete region of TRIM5α that is required for localization of the protein to cytoplasmic bodies. This region is located within the Linker2 (L2) region connecting the coiled coil domain and B30.2/PRYSPRY domain. Deletion of this region in the context of full length rhTRIM5α abrogates cytoplasmic body localization as well as retroviral restriction. Alanine mutagenesis of the residues in this region identify two stretches of amino acids that are required for both cytoplasmic localization and retroviral restriction. This work suggests that the determinants that mediate the localization of TRIM5α to cytoplasmic bodies play a requisite role in the process of retroviral restriction.

INHIBITION OF EARLY STAGES OF HIV-1 ASSEMBLY BY INI1 MUTANT S6 AND THE REVERSAL OF INHIBITION BY REDIRECTING IT TO THE NUCLEOPLASM.

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INI1/hSNF5 is an HIV-1 integrase binding protein. Previous studies demonstrated that a minimal IN-binding fragment of INI1, termed S6, selectively and potently inhibited HIV-1 particle production (Yung et al. Nat. Med. 2002 and Yung et al. J. Virol 2004). S6 contains an unmasked nuclear export signal (NES), and is cytoplasmic, while full length INI1 is nuclear (Craig et al. EMBO J 2002). Here, we have tested to determine if cytoplasmic localization of S6 is required for inhibition and have further explored the mechanism of inhibition by S6.
To determine if cytoplasmic localization of S6 is required to inhibit particle production, we generated variants of S6 with altered sub-cellular localization either by mutating NES and/or by incorporating a strong NLS. We found that targeting an NLS-containing S6 variant [NLS-S6(Rpt1)] to the nucleoplasm resulted in complete reversal of inhibition of particle production. To further explore the mechanism of inhibition, we carried out Electron microscopic (EM) and immuno-fluorescence analysis of HIV-1 producer cells expressing S6 and the non-inhibitory mutant NLS-S6(Rpt1). EM analysis indicated that while none or very few electron dense virus particles were seen in cells expressing S6, virions were produced to wild type levels in cells expressing NLS-S6(Rpt1). Immuno-fluorescence analysis revealed that while Gag accumulated along the plasma membrane in cells expressing either INI1 or NLS-S6(Rpt1), there was no membrane accumulation but a diffuse pattern of Gag localization within the cells expressing S6. Furthermore, S6 did not affect the sub-cellular distribution of Gag/GagPol when IN was deleted, demonstrating that S6-mediated inhibition was dependent on IN. Additionally, in S6-expressing cells, there was a decrease in the intracellular Gag levels. These results together indicate that ectopic expression of S6, by binding to IN, affects multiple stages including Gag synthesis or stability and accumulation of Gag/Gag-Pol along the plasma membrane, leading to inhibition of assembly at an early stage prior to particle formation and budding.

BROADLY NEUTRALIZING ANTIBODY IN EIAV-INFECTED HORSES IS DUE IN PART TO RECOGNITION OF MULTIPLE IMMUNODOMINANT NEUTRALIZING EPITOPES

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An important goal of lentivirus vaccine design is induction of broadly neutralizing antibody (bNAb). Ideally, such antibody would target conserved epitopes shared among a large number of genetically distinct primary isolates. However, broad neutralizing activity could also result from an accumulation of antibodies with multiple specificities. Horses infected with EIAV develop bNAb, which play an important role in control of virus during long-term inapparent infection. Over time, neutralization escape variants evolve and lead to recrudescence of clinical disease. A goal of our research is to identify the viral targets of bNAb and determine mechanisms of neutralization resistance. There are eight variable regions, V1-V8, in the surface envelope glycoprotein (SU) of EIAV. Two neutralizing epitopes are found in V3, in a region termed the Principal Neutralizing Domain (PND). A third neutralization epitope is found in a conserved region of V5. To investigate the role of variable regions V5, V6, and V7 in neutralization of EIAV, we used the cell culture-adapted EIAV-19 and the virulent wild-type EIAV-Wyo to generate a series of chimeric reporter viruses that differed in the PND and/or variable regions V5, V6 and V7. These viruses were tested in neutralization assays against a panel of serum collected from horses experimentally infected with EIAV-19 or EIAV-Wyo. Most sera were able to neutralize all chimeric viruses, regardless of the PND or V5-V7 genotype, indicating the presence of bNAb. In general, highest neutralizing titers were against chimeric viruses that were homologous in V5-V7; in only one horse was the homologous PND region observed to be immunodominant. Sera from two horses neutralized viruses containing either homologous or heterologous PND, but introduction of heterologous V5-V7 resulted in a loss of neutralizing activity. For these samples, the neutralization phenotype depended on the identity of the V6 region, suggesting the presence of a novel neutralization epitope in this region. Neutralizing antibody titers against chimeric viruses heterologous in both the PND and V6 were markedly reduced as compared to titers against homologous virus. Thus, there appears to be at least two distinct immunodominant neutralizing epitopes in EIAV SU, and a portion of the broadly neutralizing activity in EIAV-infected horses results from accumulation of antibodies with different specificities.

HUMAN APOBEC3G IS A MORE POTENT INHIBITOR OF HIV-1 REPLICATION THAN APOBEC3F IN ACTIVATED $CD4^+$ T CELLS AND MACROPHAGES

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Activated $CD4^+$ T cells and macrophages express APOBEC3G (A3G) and APOBEC3F (A3F), which are potent inhibitors of retroviral replication. HIV-1 Vif binds to A3G and A3F, targeting them for proteasomal degradation, thereby allowing HIV-1 replication in these cells. Distinct regions of Vif interact with A3G and A3F; $Y^{40}RHHY^{44}$ is important for binding to A3G, whereas $D^{14}RMR^{17}$ is critical for binding to A3F. In single-cycle infectivity assays, a *vif* mutant in which 5 alanines replaced $Y^{40}RHHY^{44}$ (YRHHY>A5) failed to block A3G but not A3F antiviral activity; in contrast, a *vif* mutant in which $D^{14}RMR^{17}$ was replaced with 4 alanines (DRMR>A4) failed to block A3F but not A3G antiviral activity (Russell et al. J. Virol. 81:8201,2007). Here, we used replication-competent HIV-1 YRHHY>A5 and DRMR>A4 mutants to determine the relative antiviral activity of A3G and A3F in activated $CD4^+$ T cells and macrophages by comparing their replication kinetics and the nature and extent of G-to-A hypermutation.

In the first round of infections, both YRHHY>A5 and DRMR>A4 mutants replicated in activated $CD4^+$ T cells and macrophages; only the YRHHY>A5 mutant showed a 2 - 4 day delay in replication compared to WT. In the second round of infections, initiated with virus derived from the first round, a longer 8-10 day delay in replication was observed for the YRHHY>A5 mutant, and a 2-6 day delay was observed for the DRMR>A4 mutant compared to the WT. These observations indicated that A3G is a more potent inhibitor of HIV-1 replication than A3F. The YRHHY>A5 mutant- and DRMR>A4 mutant-derived proviruses displayed G-to-A hypermutations primarily in a GG and GA dinucleotide contexts, suggesting hypermutation by A3G and A3F, respectively. Inclusion of additional mutations (E76A and/or W79A) to DRMR>A4, previously shown to diminish Vif's ability to block A3F, did not further delay the replication kinetics of the DRMR>A4 mutant.

A3G expression was higher in $CD4^+$ T cells than in macrophages. Treatment with interferon alpha (IFNα) substantially increased A3G expression in macrophages and completely abrogated virus replication; in contrast, IFNα treatment did not appreciably increase A3G expression in activated $CD4^+$ T cells, but did increase the delay in replication kinetics and hypermutation of both mutants.

In summary, we conclude that A3G and A3F both exhibit antiviral activity in macrophages and $CD4^+$ T cells and A3G is a more potent inhibitor of HIV-1 replication than A3F.

DOES THE RSV GAG PROTEIN USE PIP2 BINDING TO TARGET THE PLASMA MEMBRANE?

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The viral structural protein Gag interacts with the plasma membrane (PM) through an N-terminal membrane binding domain (MBD). In HIV-1, the MBD is composed of a myristate moiety, which can be sequestered or exposed, a phosphatidylinositol (PI) 4,5-bisphosphate [$PI_{(4,5)}P_2$)] binding pocket [1], and a highly basic region (HBR). Previous studies suggest that HIV-1 Gag binds to $PI_{(4,5)}P_2$, which causes a conformational change in the MBD that exposes the myristate moiety. This mechanism allows for specific targeting of Gag to the PM and enhances the membrane binding affinity of Gag. Reduction of $PI_{(4,5)}P_2$ levels at the PM compromises virus release and results in a re-localization of HIV-1 Gag to late endosomes [2]. A recent study suggests that RNA interactions with the HBR can also regulate membrane binding of HIV-1 Gag [3].

RSV Gag interacts with membranes through a highly basic patch [4, 5] similar to that of HIV-1 Gag, but RSV Gag is not myristoylated. We show by fluorescence microscopy that RSV Gag appears to bind to the PM by a $PI_{(4,5)}P_2$-independent mechanism. These results suggest that RSV Gag interacts with membranes only electrostatically, as shown previously for purified MA [5]. Preliminary results from liposome flotation assays of full length RSV Gag are consistent with this conclusion. We are currently assessing the effects of RNA on RSV Gag membrane binding.

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HIV-1 NEF DOWNMODULATES SOME CHEMOKINE RECEPTORS THROUGH UBIQUITINATION AND ESCRT-1 COUPLED ENDOCYTOSIS.

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It has been reported that HIV-1, HIV-2, and SIV Nef downmodulate several chemokine receptors including CCR1, CCR4, CCR2, CCR3 CXCR4, CCR5, CXCR1, CXCR2, and CXCR4. We show here that HIV-1 Nef downmodulates a tested set of chemokine receptors to a variable extent based on the cell type, receptor, and primary cell donors. Of all the chemokine receptors examined, CXCR4 in different primary cells and tumor cell lines and CXCR1 and CXCR2 in certain cell lines were downregulated better by different HIV and SIV Nef alleles in the context DNA transfection, single-cycle HIV infection or Nef protein transduction, although the downregulation was quite modest compared to that of CD4. Previous studies have mapped the domains of CXCR4 and Nef required for the down regulation of this receptor. However the understanding of the mechanisms involved is limited. In general the Agonist bound receptors undergo phosphorylation recruiting E3 ligase, which ubiquitinates (Ubq) CXCR4. The Ubq residues serve as endolysosomal sorting signals through the ESCRT pathway. We hypothesized that the HIV-Nef serves as an intracellular agonist surrogate for CXCR4 and other chemokine receptors, recruiting AIP4/NEDD4 E-3 ligases to ubiquitylate CXCR4 followed by a dynamin dependent endocytosis into MVBs for proteolyis. This mechanism was confirmed by interaction between Nef, AIP4 and CXCR4 and by reversal of Nef effect through siRNA knockdown of AIP4 or by a small molecular weight inhibitor of dynamin. We also show that both naturally truncated mutants of CXCR4 as in WHIMS or engineered truncated variants of CXCR2 and CXCR4 (which lack the classical motifs for endocytosis and ubiquitylation) are downregulated more profoundly than wt receptors, probably through direct recruitment of AP2 adapter. Thus, we demonstrate here that the mechanism of Nef induced CXCR4 internalization follows the general paradigm of agonist occupied chemokine receptors.

AUTOPROCESSING OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 PROTEASE MINIPRECURSORS FUSED TO GLUTATHIONE TRANSFERASE IN MAMMALIAN CELLS

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HIV protease (PR) is a virus-encoded aspartic protease that is essential for viral replication. The fully active and mature dimeric protease is produced from the Gag-Pol polyprotein as a result of autoprocessing. We here describe a cell based assay examining HIV protease autoprocessing in transfected mammalian cells. A well characterized miniprecursor consisting of the mature protease along with its upstream transframe region (TFR) was in-frame fused to the C-terminus of GST and the resulting chimeric precursors were able to autoprocess releasing mature protease in E. coli and HEK 293T cells. Protease inhibitors Indinavir and Duranavir suppressed autoprocessing when transfected cells were incubated with the inhibitors. To further evaluate the mammalian expressed GST fusion miniprecursors for their utility, we constructed mutants that were previously characterized with other model systems and examine their protease autoprocessing in transfected 293T cells. These mutants displayed previously reported phenotypes including: 1) H69E mutation in a pseudo wild type protease backbone reduced autoprocessing; 2) the H69E inhibitory effect was partially rescued by A95C mutation; 3) H69D suppressed autoprocessing more than H69E in NL4-3 protease sequence. With this system, we also demonstrated that N-terminal truncations of TFR, up to 46 out of a total of 54 residues, do not affect protease autoprocessing. Consistent with this, replacing the TFR with an unrelated peptide (55 amino acid long derived from the N-terminus of Hect1 protein) while keeping the cleavage site between TFR and PR had no impact on protease autoprocessing. Taken together, we suggest that the mammalian expressed GST fusion miniprecursor is a simple and useful model to study protease autoprocessing, and it can be further developed for cell-based screening of new drugs that interfer with protease autoprocessing.

FLUORESCENCE SUBDIFFRACTION MICROSCOPY OF HIV-1 ENV DISTRIBUTION ON FREE AND CELL-ATTACHED VIRIONS.

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HIV-1 envelope protein (Env) is a trimer of heterodimers (gp120/gp41) that is responsible for a virus attachment and fusion. Individual virions contain a low number of Env trimers with some studies indicating the requirement of a concerted action of several Env trimers for entry. Despite the detailed knowledge about HIV-1 Env structure and its fusion mechanism, not much is known about the actual distribution of Env on individual particles and their rearrangements on the virus surface during the early stages of attachment and entry. Stimulated Emission Depletion microscopy (STED) is a subdiffraction fluorescence microscopy technique that can resolve biomolecules at a resolution of up to 20 nm thus offering a unique possibility to study subviral structures by a fluorescence microscopy. Here, we have used STED microscopy to observe HIV-1 Env distribution on wild-type (wt) and mutant virions and to detect Env rearrangements upon CD4 receptor engagement. Mature wt HIV-1 particles predominantly displayed a clustering of Env molecules, with the majority of virions showing only a single Env cluster. This was different for immature HIV-1 particles which revealed a broader, multi-clustered distribution. A similar phenotype was observed when the immature Gag lattice was stabilized by a mutation of cleavage sites or when the C-terminal tail of Env was truncated. These results indicate that a virion maturation affects the outer surface of the particle and also alters Env lateral motility dependent on the Env C-terminal tail. These findings were correlated with a parallel cryo-electron tomography analysis of Env distribution. We have also analyzed rearrangements of Env molecules upon the receptor engagement by determining Env colocalization with CD4 patches on the cell membrane for wt and mutant HIV-1. These experiments indicate a maturation-dependent differences in Env distribution on the virion surface and its redistribution during the receptor interaction which may play a role in HIV-1 entry.

THE HTLV-1 HBZ ANTISENSE GENE PROMOTES TAX EXPRESSION

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Human T-cell leukemia virus type 1 (HTLV-1) basic leucine zipper factor (HBZ) is transcribed from the antisense genomic DNA strand and functions differently in its RNA and protein forms. To distinguish between the roles of *hbz* mRNA and HBZ protein, we generated mutants in a proviral clone that specifically disrupt the *hbz* gene product. A proviral clone with a splice acceptor mutation that disrupts expression of the predominant *hbz* mRNA resulted in lower levels of *tax* mRNA, as well as lower levels of Gag proteins. Heterologous *hbz* expression restored Tax activity to cells expressing this mutant clone. In contrast, proviral mutants that disrupt HBZ protein but not *hbz* mRNA did not affect levels of *tax* mRNA. Since *hbz* is a natural antisense RNA to genes expressed from the (+) genomic DNA strand, we hypothesized that *hbz* mRNA represses an inhibitor of *tax*. $p30^{II}$, an accessory viral protein, has been shown to down-regulate *tax* expression, and since the majority of the *hbz* transcript is complementary to the $p30^{II}$ transcript, we suggest that *hbz* promotes expression of *tax* by down-regulating $p30^{II}$ expression. Mutation of $p30^{II}$ overcame the effects of the splice acceptor mutation of *hbz*, and restored *tax* expression. Additionally, levels of $p30^{II}$ mRNA were reduced in the presence of HBZ. Thus, there is a complex interplay of viral regulatory proteins controlling levels of HTLV-1 gene expression.

RNA FEATURES AND POTENTIAL HOST FACTORS INVOLVED IN MRNA TRANSLATION AND GRNA PACKAGING OF THE YEAST RETROTRANSPOSON TY3.

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When expressed in mating populations of *S. cerevisiae*, the retrotransposon Ty3 utilizes mRNA for both the template for translation and the packaging of genomes into virus-like particles (VLPs). As there is no separate pool of Ty3 genomic (g)RNA used solely for packaging into VLPs, translation and selection for packaging are likely to be inherently linked. However, the point at which commitment to packaging occurs for Ty3 is not known. Ty3 RNA and proteins co-localize with cellular proteins involved in RNA processing, degradation, and translation regulation. Additionally, while 5' untranslated regions (UTRs) of Ty3 are predicted to contain stable secondary structure motifs important for identifying transcripts for packaging – as is the case for retroviruses – the presence of these structures could restrict translation initiation and ribosome scanning. Regions of Ty3 RNA were investigated to determine the features required for gRNA dimerization and packaging. Our results show that UTRs are not required for localization of *GAG3-POL3* RNA with P-body proteins but do contribute significantly to packaging. Preliminary analysis of native *GAG3* RNAs suggests that other features apart from UTRs may contribute to the formation of *GAG3* RNA dimers *in vivo*. Ty3 RNA that has been packaged into VLPs maintains a 5' cap structure, indicating that RNA decapping is not a requirement for Ty3 gRNA packaging. We also investigated the role of host cell proteins in committing a Ty3 RNA for translation or packaging. In a genome-wide screen of host factors required for transposition, the P-body protein and DEAD-box helicase Dhh1p and DEAD-box helicase Dbp3p were identified. As DEAD-box family proteins have been shown to be global regulators of translation and RNA processing, and can also be constituents of P bodies, the roles of these proteins in Ty3 replication were investigated. Deletion of *DHH1* resulted in a dramatic decrease in Ty3 RNA and protein during alpha factor- and galactose-induced Ty3 expression. Over-expression of Dhh1p also decreased Ty3 protein levels. Deletion of *DBP3* resulted in a lesser effect on Ty3 RNA and protein levels, and WT levels of cDNA production, but produced an equally dramatic decrease in transposition as that of the *DHH1* deletion. Thus, Dhh1p appears to act on the Ty3 lifecycle at the translational level, potentially by modulating the switch between RNA degradation/packaging and translation. Contrastingly, the absence of Dpb3p results in a phenotype consistent with a post- translational, pre-integration defect.

THE HIV-1 ASSEMBLY COMPARTMENTS IN PRIMARY MACROPHAGES ARE PART OF THE ENDOCYTIC NETWORK AND ARE ENRICHED IN TETHERIN

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HIV-1 assembles and buds at the plasma membrane of T cells and most epithelial cell-lines, whereas in macrophages, HIV-1 particles are predominantly found in intracellular compartments. Some recent reports suggest that these intracellular compartments are in fact modified plasma membrane compartments that are continuous with the cell surface. In this study, we present several lines of evidence that show that these compartments are endocytic in nature and describe a potential role of tetherin in the formation of these compartments. With immunofluorescence and electron microscopic methods, we demonstrated that the majority of the virus-containing compartments (VCCs) in HIV-1 infected macrophages were not accessible from the plasma membrane. We further found that the VCCs were intensely enriched in tetherin, which is a HIV-1 restriction factor that normally recycles through the cellular endocytic network. Next, we examined the endocytosis of tetherin by pulse-labeling cell surface tetherin. We found that tetherin was rapidly endocytosed into the VCCs from the plasma membrane and at where it was retained together with the HIV-1 particles. Altogether, our data suggest that the viral compartments in infected macrophages are part of the endocytic pathway, and are highly enriched in tetherin. The rapid endocytosis of tetherin, together with its known ability to retain HIV-1 particles, and its co-enrichment in the intracellular endosomes with HIV-1 further suggests that it plays a role in sequestering the virus in these intracellular sites.

RNA BOUND TO THE HIGHLY BASIC REGION OF HIV-1 MATRIX INHIBITS GAG-MEMBRANE BINDING

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HIV-1 assembly is mediated by the polyprotein precursor Gag that contains four major structural domains: matrix (MA), capsid (CA), nucleocapsid (NC) and p6. One of the most crucial steps in HIV-1 assembly is membrane binding of Gag. This step is facilitated by bipartite signals of MA: N-terminal myristoylation and the highly basic region (HBR) between amino acids 17 and 31. The N-terminal myristate is normally sequestered into the MA globular domain, and a structural change exposes myristate and enhances membrane binding. The HBR, on the other hand, facilitates membrane binding by interacting with an acidic lipid, phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2]. Additionally, analyses of liposome binding of Gag synthesized in vitro using rabbit reticulocyte lysates suggest that the HBR can also suppress PI(4,5)P_2-independent membrane binding by keeping the myristate sequestered.
Membrane binding of Gag is also enhanced by Gag multimerization. In addition to CA dimerization, RNA bound to NC can act as a scaffold and bring Gag molecules together. To understand the role of RNA in Gag-membrane binding, we treated Gag synthesized in vitro with RNase before mixing with liposomes. To our surprise, RNase-treated Gag binds liposomes much more efficiently compared to non-treated Gag suggesting that RNA inhibits membrane binding. Mutational study suggests that the RNA bound directly to MA HBR but not NC is involved in this regulation of membrane binding. Further analysis indicates that RNA inhibits electrostatic interaction between HBR and acidic lipids. In addition, enhanced binding of RNase-treated Gag to neutrally-charged liposomes suggest that RNA can also modestly inhibit myristate exposure. Preliminary results show that MA HBR interacts with RNA not only in the in vitro system but also in cells. Currently, we are investigating which RNA is bound to the MA HBR and whether it inhibits membrane binding in cells. In summary, RNA bound to the MA HBR inhibits membrane binding by blocking both electrostatic interaction with acidic lipids and myristate exposure and may represent a novel regulator of Gag-membrane binding.

REVERSE TRANSCRIPTASE MUTATION K65N CONFERS A DECREASED REPLICATION CAPACITY TO HIV-1 IN COMPARISON TO K65R DUE TO A DECREASED RT PROCESSIVITY

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In addition to K65R, the other mutation that is observed at RT codon 65 is K65N. This mutation is rarely observed during clinical trials. The <I>in vitro</I> selection of this mutation along with K70R has been shown to occur during the passaging of HIV-1 LAI with the combinations of emtricitabine (FTC) and tenofovir (TDF). Also, in vivo selection of this mutation in patients with M184V or non nucleoside reverse transcriptase mutation has been demonstrated with abacavir-lamivudine-TDF or FTC-ddI-efavirenz treatments. The development of K65N with other mutations was attributed to the variable drug concentrations. We hypothesized that viruses containing K65N mutation will be attenuated in comparison to viruses with K65R change.

In order to assess the relative impact of K→N with respect to K→R change, we performed site-directed mutagenesis to insert K65R and K65N in proviral clone pNL4-3. Viruses were generated upon transfection of 293T cells and replication kinetics assays were performed in PHA-stimulated PBM cells and MT-2 cells in the absence of drug. We found K65N has a significant loss in replication capacity in comparison to K65R ($p = <0.011$). In order to understand the biochemical mechanisms involved in viral attenuation, in vitro RT processivity was performed using virion-associated RT with homopolymer poly (rA) and oligo (dT) primer in the presence of radio-labelled dNTP (α-³²P TTP) and trap poly (rC)-oligo (dG). A significant decrease both in the length of cDNA ($p = 0.001$) and the density of cDNA ($p = 0.0001$) synthesis was observed with K65N RT in comparison to K65R RT. Since our previous studies have shown that K65R+L74V mutant is highly crippled and the initiation of replication of this virus leads to R→K reversion, we performed amino acid polymorphism analysis of RT codon 65 and created various mutants singly or in the background of L74V mutation: K65A, K65E, K65Q, K65I, K65T K65N+L74V, K65A+L74V, K65E+L74V, K65Q+L74V, K65I+L74V, K65T+L74V. Replication kinetics assays showed absence of any measurable RT activity in PBM cells. A low level of replication was observed in MT-2 cells but the viruses from MT-2 cells failed to initiate any replication in PBM cells. Comparison of RT processivity between doubly mutants K65R+L74V and K65N+L74V showed a significant decrease in single cycle RT activity ($p = 0.001$) and processivity ($p = 0.05$) of latter RT.

Our observations suggest that due to slower growth kinetics of K65N viruses in comparison to K65R, the selection of the K65R mutation may be favored in clinical use of antiretroviral drugs compared to K65N. The deleterious effect on viral replication by various changes at RT codon 65 could not be compensated by the addition of L74V on the same viral genome. An anti-retroviral therapy regimen enforcing the selection of K65N and L74V on the same viral genome may be a useful strategy for the management of HIV disease.

ADAR1 AND PACT INHIBIT PKR ACTIVATION DURING HIV INFECTION OF LYMPHOCYTES

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The interferon-induced protein kinase RNA-activated (PKR) is activated after virus infection. This activation is only transient during human immunodeficiency virus (HIV) infection of lymphocytes and the protein is not activated at the peak of infection. We observed that the interferon-induced Adenosine deaminase acting on RNA (ADAR)1-p150 and ADAR1-p110 expression increases while the virus replicates actively. Furthermore, both forms of ADAR1 and the PKR activator (PACT) show enhanced interactions with PKR at the peak of HIV infection suggesting a role of these proteins in the regulation of PKR activation. We observed that ADAR1p150 reverses PKR inhibition of HIV virus expression and production in HEK 293T cells. This activity requires the Z-DNA binding motif and the three double-stranded RNA binding domains, but not the catalytic domain. Surprisingly, PACT also reverses PKR inhibition of HIV virus expression and production in HEK 293T cells suggesting a role of PACT as a PKR inhibitor. These results indicate that two interferon-induced proteins, ADAR1 and PKR can have antagonistic functions. They also suggest that PACT switches from a PKR activator to a PKR inhibitor in the context of active HIV replication.

INVESTIGATING HIV-1 CIS AND TRANS PACKAGING ELEMENTS THROUGH RECONSTRUCTION IN A SIMPLE RETROVIRAL VECTOR

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Encapsidation of viral RNA into a HIV-1 particle requires interactions between *cis* elements localized primarily in the 5' UTR of the viral RNA and *trans* factors encoded by the virus, & host cell genome. The HIV-1 Rev protein was recently shown to influence encapsidation of HIV-1 RNA in the presence of the major *cis* packaging elements. Conceivably, the encapsidation functions attributed to *cis* elements in the 5' UTR and the Rev/RRE system are separable. We investigated whether a gain of encapsidation function could be achieved through transfer of HIV *cis* elements to a heterologous RNA. The HIV-1 RRE and major *cis* packaging determinants (HIV-1 R, U5, PBS, & canonical packaging signal) were systematically incorporated into a murine leukemia virus (MLV) vector RNA to assess encapsidation into HIV-1 viral particles. Chimeric HIV-1 particles harboring MLV RNA can transduce cells, thereby supporting a rapid method for assessing encapsidated RNA into viral particles by titering for a GFP reporter. In an MLV vector, devoid of all conventional HIV-1 *cis* packaging elements, the Rev/RRE enhanced encapsidation of the MLV vector RNA 5-10 fold into a HIV-1 viral particle. Encapsidation was augmented nearly an additional 100-fold by incorporating all HIV-1 major *cis* packaging determinants into the middle of the MLV vector. *Cis* elements consisting of both the R-U5-PBS, and conventional packaging signal, are necessary to achieve a synergistic encapsidation effect in the presence of the Rev/RRE. Rev/RRE-mediated nuclear export of RNAs through the CRM-1 pathway may impact the capacity to enhance encapsidation. Employing a previously characterized RevM10-Tap fusion protein to redirect RRE-containing vector RNAs from the CRM-1 to the Tap-dependent nuclear export pathway we demonstrate that encapsidation with the RevM10-Tap system was comparable to that obtained with conventional Rev. We provide evidence showing that i) Rev can enhance encapsidation of heterologous RNA independent of a conventional HIV-1 packaging signal; ii) the R, U5, PBS, & canonical packaging signal dramatically enhance encapsidation upon transfer to a heterologous RNA; iii) *cis* elements in the entire 5' UTR and the Rev/RRE system function synergistically to support RNA encapsidation specifically into HIV-1 particles; and, iv) Rev/RRE dependent encapsidation can be accomplished through CRM-1, or Tap dependent nuclear export.

INTERFERON-ALPHA ACTIVATION OF DENDRITIC CELLS RESTRICTS HIV-1 INFECTION AND TRANSMISSION: A POTENTIAL ROLE OF TETHERIN?

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Dendritic cells (DCs) have close interactions with CD4+ T cells, the main targets of HIV replication. DC subsets have been investigated for their role in transmission of captured HIV from the site of infection to the main T cell pool. Immature DCs (iDCs) and mature DCs (mDCs) transmit HIV-1 to cocultured CD4+ T cells with different efficiencies. Various stimuli including lipopolysaccharide (LPS) and interferons (IFN) have been used to induce DC activation. DC-mediated HIV-1 transmission can be regulated by host factors. Tetherin (CD317 or BST-2) is a type-1-IFN-induced cellular protein that restricts HIV-1 particle release. Tetherin acts as an important component of the innate defense against several other enveloped viruses. The potential role of tetherin in DC-mediated HIV-1 transmission remains unknown. To explore the potential role of tetherin in DC-mediated cell-to-cell transmission and replication of HIV-1, monocyte-derived iDCs, mDCs induced by LPS (mDC-LPS) and IFN alpha (mDC-IFN-α) were compared for tetherin expression and HIV-1 replication and transmission efficiency. DC surface and total expression of tetherin was assessed by flow cytometry and immunoblotting, respectively. iDCs were negative for tetherin, while mDCs-LPS were highly positive in both measures; and mDC-IFN-α were partially positive by flow cytometry and highly positive by immunoblotting, indicating efficient up-regulation of intracellular, but not surface tetherin expression by IFN-α. Interestingly, mDC-IFN-α displayed significantly reduced capacity of HIV-1 uptake and transmission compared to mDC-LPS, and reduced HIV-1 replication compared to iDCs. Furthermore, mDC-IFN-α showed unique chain-like localization of HIV-1 particles on the cell surface, suggesting altered viral trafficking by IFN-α activation. The potential role of tetherin in mDC-LPS-mediated HIV transmission is being examined by silencing tetherin expression using specific siRNA. The mechanisms of mDC-IFN-α restrictions of HIV infection and transmission are under investigation. These studies will help to further understand the mechanisms of cell-to-cell transmission of HIV-1.

VIRAL SUPPRESSION OF RNAi: TRBP AS A TARGET OF HIV ELEMENTS

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RNA interference (RNAi) regulates gene expression in eukaryotes. The assembly of a ribonucleoprotein complex, the RNA-Induced Silencing Complex (RISC), is crucial for RNAi activity. In addition, many organisms use RNAi as an antiviral defence mechanism. Although it is still unclear whether or not this is the case in humans, several viruses have been shown to suppress RNAi activity.

One key protein of the human RISC is the TAR-RNA Binding Protein (TRBP). TRBP was characterized for its ability to bind to the Human Immunodeficiency Virus (HIV) TAR RNA, and is required for productive viral replication. This work explores the relationship of TRBP to RNAi and HIV replication to determine whether elements of HIV are able to inhibit RNAi activity in the cell.

HIV has been suggested to inhibit RNAi by sequestering TRBP from the RISC through the TAR RNA element. We tested the ability of this and other HIV-encoded elements to inhibit RNAi activity. Our results indicate that certain HIV elements interact with TRBP and restrict RNAi.

RNAi has emerged as a critical regulator of cellular functions. The ability of HIV components to disrupt RNAi activity may have important implications in both pathogenesis and treatment options at a time when RNAi-based gene therapy is becoming an attractive new tool for the control of infectious diseases.

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INTERPLAY OF HIV-1 FITNESS AND MUTATION RATE

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Understanding HIV-1 population dynamics is essential to better predict viral disease progression to AIDS, durable antiretroviral drug regimens, and for the development of an effective vaccine. HIV-1 populations are best described as a quasispecies. In this study, we investigated a fundamental feature of HIV-1 quasispecies formation by exploring the relationship between viral fitness and mutation rate. We propose a model in which optimal viral fitness corresponded to the defined optimal viral mutation rate, and reductions in viral fitness would coincide to either increases or decreases in viral mutation rate. In order to experimentally test our model, we have created a set of HIV-1 mutants, most of which contain single amino acid mutations that confer drug-resistance to nucleoside reverse transcriptase analogs. These mutants were used in parallel assays to assess viral fitness, mutation rate and rate of viral DNA synthesis. Our findings support our model of the relationship of viral fitness and mutation rate and this study represents the first comprehensive analysis of such mutants. A prediction made from our model and our studies is that purposeful increases or decreases in viral mutation rate can result in reductions in viral fitness – an outcome that would have therapeutic applications.

DEVELOPMENT OF HIGHLY SENSITIVE ASSAYS FOR THE DETECTION OF XMRV NUCLEIC ACIDS IN CLINICAL SAMPLES

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Xenotropic murine leukemia virus-related virus (XMRV) is a new human retrovirus first identified in prostate cancer tissues from men with a subtle deficiency in an innate immunity gene. Currently, in different studies, 0 to 27% of prostate cancer cases and 0 to 67% of chronic fatigue syndrome (CFS) patients showed evidence of XMRV infections. While the explanation for these different results is unknown, differences in genetics, geographical distribution, sequence diversity in viral strains, or methodological variations are suspected. Here we will describe our methods for detecting low copies of XMRV nucleic acids and the challenges we have encountered. Various clinical samples were obtained from prostate cancer patients just prior to and following radical prostatectomy. Prior to surgery, prostate massage was performed and urine samples were collected containing prostatic secretions. Flash frozen prostate tissues from the peripheral zones were obtained after radical prostatectomy. In addition, expressed prostatic secretions (EPS) were obtained by manually milking the prostate after surgery. RNA (or DNA) were extracted from the samples and real-time RT-PCR (or PCR) were used to measure low copies (as few as 10) of XMRV RNA or DNA. Confirmation of the presence or absence of XMRV nucleic acids was obtained by nested RT-PCR (or PCR). These methods specifically amplify env sequences corresponding to variable regions A & C of the gp70 envelope protein. The identity of the amplicon was confirmed by DNA sequencing. Results show less than 100 copies of XMRV DNA was present per microgram of total DNA from prostate tissue. Similarly, RNA isolated from patient samples contained 500 or fewer copies of XMRV RNA per microgram of total RNA. These low levels of XMRV nucleic acids in patient samples highlight the challenges of performing PCR methods as an indicator of XMRV infections. However, once fully developed, PCR methods may lead to large-scale high throughput assays for monitoring XMRV in prostate cancer patients, in other diseases, and in the general population.

HIV-1 CAPSID AND THE EARLY STEPS OF INFECTION LEADING UP TO INTEGRATION

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HIV-1 infects non-dividing cells. This implies that the virus traverses the nuclear pore before it integrates into chromosomal DNA. Recent studies characterizing HIV-MLV chimeras and HIV-1 Capsid (CA) mutants suggest that CA plays a critical role in nuclear entry of the viral preintegration complex (PIC). To shed light on the mechanism by which CA contributes to this essential step in HIV-1 infection we have engineered a panel of single-cycle HIV-1 vectors bearing CA mutations that have been partially characterized in previous studies. These mutants will be extensively characterized using standard methods for virion assembly, capsid stability, virion infectivity, synthesis of viral cDNA, 2-LTR circles, and proviral DNA. Infectivity of the mutants will be analyzed under conditions of cell cycle arrest, and knock-down of CA interacting proteins. At the same time we are attempting to develop new methods for visualizing PICs in fixed and living cells; these methods will be used to better characterize the replication defects of the above-mentioned CA mutants.

CHARACTERIZATION OF VPR (52-96) ENTRY AND BEHAVIOUR IN CELLS

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Vpr is a 96 residue basic protein that impacts on the cell physiology during HIV replication [1]. The protein is detected in the blood serum of infected patients and enters non-infected cells causing G2/M cell cycle arrest and apoptosis [2]. Rhodamine and fluorescein derivative of the Vpr C-terminus were used to describe the mechanism of Vpr(52-96) cellular uptake. The clathrin-coated pits or the caveolae pathways were followed by transferrin and methyl-β-cyclodextrin suggesting an energy dependent uptake of Vpr(52-96) but a fraction of peptide could translocate at 4°C. Vpr C terminus forms large aggregates that interact with the PM even though the inhibition of macropinocytosis by N,N-dimethylamylorid did not limit the uptake. In the cell, Vpr(52-96) diffuses in the cytoplasm and in the nucleus while aggregates stain in late endosomes/lysosomes and are highly stable as monitored by FRET based images [3]. Moreover, we show a direct interaction with lipids that destabilize the bilayer rather than inducing channel segments.

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FIRST-IN-CLASS INHIBITORS OF LEDGF/P75-INTEGRASE INTERACTION AND HIV REPLICATION (LEDGINS)

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Background: In 2003 we identified the cellular protein, LEDGF/p75, as a strong binding partner of HIV-1 integrase (IN) in eukaryotic cells. We have since established a thorough validation procedure for novel HIV co-factors. Implementation of our validation scheme corroborated LEDGF/p75 as an important co-factor of HIV integration and validated the virus-host interaction as a potential drug target for antiviral therapy.
Methods: The resolved X-ray structure of IN-core complexed with the IBD of LEDGF/p75 revealed a well defined interphase suggesting that small molecule protein-protein interaction inhibitors (SMPPII) might be capable of disrupting the interaction. We have now applied rational drug design to identify small molecules potentially binding to the interaction site. For in vitro hit identification we developed a high throughput Alphascreen assay monitoring the LEDGF/p75 HIV-1 Integrase interaction. Antiviral activity and cytotoxicity of initial hits from in vitro screening were evaluated in cell culture in the MT4/MTT HIV replication system.
Results: We have rationally designed a series of 2-(quinolin-3-yl)acetic acid derivatives that act as potent inhibitors of the LEDGF/p75-integrase interaction and HIV-1 replication at submicromolar concentration by blocking the integration step. A 1.84 Å resolution co-crystal structure corroborates the binding of the inhibitor in the LEDGF/p75 binding pocket of integrase. This structure and the lack of cross-resistance with two clinical integrase inhibitors (Raltegravir and Elvitegravir) defines the 2-(quinolin-3-yl)acetic acid derivatives as the first genuine allosteric HIV-1 integrase inhibitors.
Conclusion: Our work demonstrates the feasibility of rational design of small molecules inhibiting the protein-protein interaction between a viral protein and a cellular host factor.
The discovery of the 2-(quinolin-3-yl)acetic acid derivatives as the first inhibitors of LEDGF/p75-IN interaction (LEDGINs) and HIV replication provides the ultimate proof for the crucial role of the cofactor in HIV replication. The antiviral profiling as well as their ADMEDTox profile demonstrate their potential for antiviral therapy.

INVESTIGATION INTO THE MECHANISTIC IMPLICATION OF TNPO3 AND THE CENTRAL DNA FLAP IN HIV-1 NUCLEAR IMPORT

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The active nuclear import of HIV-1 through the nuclear pore accounts for its ability to replicate in non-dividing cells. Despite many papers published on the topic, it remains entirely unknown how the HIV-1 DNA genome achieves translocation through the nuclear pore. It is assumed that it is compacted, protected and rendered karyophilic by viral and possibly cellular proteins that associate with it. Indeed, viral nuclear import factors have been proposed, such as integrase (IN) and the central DNA Flap, however their cellular interaction partners remain unknown. Transportin-SR2 (TNPO3) is a karyophilic shuttle protein shown to be involved in HIV-1 replication and nuclear import. Although TNPO3 was originally identified as binding partner for IN, the identity of the physiological viral interaction partner for TNPO3 is currently under debate. We therefore sought to determine the mechanistic implication of TNPO3 in HIV-1 nuclear import. We were intrigued to note that the Saccharomyces cerevisiae orthologue of TNPO3, MTR 10, shuttles tRNAs from the cytoplasm back into the nucleus, and hypothesised that TNPO3 might act as an interaction partner for the single stranded DNA Flap that is present at the centre of the HIV-1 genome following reverse transcription. To test this hypothesis, we infected knock-down Hela cells for TNPO3 with HIV-luc-VSV-G viruses with wild type or 225T Flap mutant. As previously published, we observed a ~10-fold reduction in infectivity in the absence of TNPO3, and a ~10-fold reduction in infectivity in the absence of the central DNA Flap. However, the enhancement of infectivity brought by the presence of DNA Flap was not lost upon TNPO3 knock-down, indicating that the central DNA Flap still confers an infection advantage in the absence of TNPO3. Therefore, we conclude that TNPO3 does not interact with the central DNA Flap to promote nuclear import. Interestingly, CA point mutations have been reported that render HIV-1 insensitive to TNPO3 knock-down, and work is undergoing to investigate whether TNPO3 might interact functionally or physically with HIV-1 CA.

EVOLUTION OF THE ANTIRETROVIRAL RESTRICTION FACTOR TRIMCYP IN OLD WORLD PRIMATES

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TRIMCyp is a fusion protein related to the prototype restriction factor TRIM5α, which restricts retroviral replication at a post-entry step. TRIMCyp evolved independently in Old World and New World monkeys, representing a striking example of convergent evolution. Old World TRIMCyp has been reported in three species of Asian macaques, *Macaca nemestrina, M. mulatta*, and *M. fascicularis*. Because *M. nemestrina* belongs to the earliest diverging branch of Asian macaques, TRIMCyp must have evolved before the radiation of Asian macaques. However, the only other Old World primate species tested for TRIMCyp is the sooty mangabey, an African monkey, which was found to lack TRIMCyp. To further define the evolutionary origin of Old World TRIMCyp, we examined two species of baboons and three additional macaque species. *M. sylvanus* is the only macaque species found outside Asia, and represents the earliest diverging branch of the macaque lineage. Baboons and sooty mangabeys belong to a sister clade to macaques. Old World TRIMCyp expression requires the presence of a cyclophilin A (CypA) pseudogene retrotransposed into the 3' untranslated region of the TRIM5 gene, and of a single nucleotide polymorphism (SNP) that allows alternative splicing to the CypA transcipt. Neither TRIMCyp expression, nor the CypA pseudogene, nor the SNP was found in baboons or *M. sylvanus*. We conclude that TRIMCyp evolved in macaques approximately 5-6 million years ago, after the divergence of Asian macaques from *M. sylvanus* but before the radiation of Asian macaques. This likely occurred as a result of retroviral threat(s) to the ancestors of Asian macaques. We also tested single individuals from two additional Asian macaque species, *M. nigra* and *M. thibetana*. Both animals were homozygous for the absence of the CypA insertion and of the SNP associated with the TRIMCyp allele. This indicates that at least some individuals of both of these species lack the capacity for TRIMCyp expression. Because TRIMCyp is fixed in *M. nemestrina*, which is closely related to *M. nigra*, this fixation must have occurred after these species diverged, approximately 2 million years ago. The macaque lineage is unique among primates studied so far due to the presence and diversity of TRIM5 and TRIMCyp restriction factors. Studies of these antiviral proteins may provide valuable information about natural antiviral mechanisms, and give further insight into the factors that shaped the evolution of macaque species.

THE ROLE OF E3 UBIQUITIN LIGASES IN HTLV-1 RELEASE

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A number of studies have demonstrated that Nedd4 (neuronal precursor cell-expressed developmentally down-regulated-4) family ubiquitin ligases (ULs) are involved in virus release via interacting with the late domains of retroviral Gag proteins. Nedd4 family E3 ULs contain nine members in humans including Nedd4, Nedd4L, NEDL1 (BUL1), NEDL2 (BUL2), WWP1, WWP2, ITCH, SMURF-1 and SMURF-2. HTLV-1 Gag has two late domain motifs, PPPY and PTAP. Previous studies by us and by the other groups have shown that the PPPY motif is more important for HTLV-1 release than the PTAP motif and that the PPPY motif interacts with WW domains of E3 ULs. Mutations of the PPPY late domain motif of HTLV-1 arrest release of virus particles from cells at an early stage of budding. We found that one particular Nedd4-like E3 UL, called ITCH could overcome the block in virus release of HTLV-1 mutants where the PPPY motif was changed to APPY or PPPF. Electron microscopic analyses showed that these PPPY-mutant viruses budded with normal morphology in the presence of ITCH and infectivity studies demonstrated that the specific infectivity of these virus like particles was close that of wt particles. In contrast, PPPY mutants PPPG and PPPD could not be rescued. Surprisingly, we also found that another UL, SMURF-1, restored virus budding to APPY mutants but that the resulting particles were not infectious. Furthermore, SMURF-1 completely inhibited the infectivity of HTLV-1 WT and PTAP mutant, but had no effect on virus release. Catalytically inactive mutants of ITCH and SMURF-1 showed no effects on the infectivity and release of HTLV-1.

IDENTIFICATION OF THE FUNCTIONAL DOMAINS AND CELLULAR BINDING PARTNERS OF HUMAN T-CELL LEUKEMIA VIRUS TYPE 2 P28 PROTEIN

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The human T-cell leukemia virus type 2 (HTLV-2) expresses a p28 accessory protein that negatively regulates viral replication. p28 in encoded by open reading frame (ORF) II from a singly spliced mRNA and is localized to the nucleus. p28 has been found to act post-transcriptionally by binding tax/rex mRNA and retaining it in the nucleus thereby reducing the amount of Tax. The negative regulation of Tax, although leading to reduced protein expression and reduced virion production, was found to be essential for viral persistence in the in-vivo rabbit model of HTLV-2 infection. HTLV-1 encodes a related p30, which acts similarly to p28 in a post-transcriptional manner to repress viral replication. However, p30 also acts in a transcriptional manner to modulate viral replication, an activity not apparent in p28.

In the present study we aim to elucidate the mechanism of p28 repressive activity by understanding the role of the functional domains of p28, the effect of post-translational modifications on p28 function and biochemical properties, and the potential contributions of the host proteins on p28 function. We generated a panel of p28 deletion mutants and tested their ability to repress Tax function in a reporter assay. Our preliminary data shows that the last 60 amino acids of p28 are not required to repress Tax function, and the region 100-216 might be exercising an inhibitory role on p28 function. Interestingly amino acids 1-50, which share homology to the c-terminus of p30/p13 of HTLV-1, can still cause repression of Tax in our reporter assay. In addition, we evaluated post-translational modifications on p28 and identified 6 serines and 1 tyrosine that are phosphorylated in 293T cells. The functional role of these residues will be discussed. Finally, we identified several cellular binding partners to p28 using proteomics analysis. We are in the process of validating the interactions and the overall contribution of these interactions to p28 function and results will be presented. This study will provide further insight into the mechanism of action of the HTLV-2 p28 accessory gene product and will allow us to better understand its role in viral infectivity, regulation of viral gene expression, and persistence.

MULTIPLE FACTORS AFFECT MMTV REM PROTEIN PROCESSING AND FUNCTION

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Mouse mammary tumor virus (MMTV) encodes a regulatory protein (Rem). Rem expression, similar to other Rev-like retroviral proteins, increases nuclear export of genome-length viral RNA. Rem also has a post-export function that requires the presence of the Rem-responsive element (RmRE), which spans the junction of the envelope gene and the 3' LTR. Our previous data have shown that Rem has a 98-amino-acid long signal peptide (SP). Rem contains all of the Rev-like domains, including a nuclear/nucleolar localization sequence, an RNA-binding motif, and a nuclear export sequence. Recent results suggest that Rem is cleaved by signal peptidase to yield SP and a C-terminal glycosylated product. SP activity can be detected using a luciferase reporter vector based on the 3' end of the MMTV genome, including the RmRE. Furthermore, expression of SP alone is functional in the reporter assay, whereas expression of the Rem C-terminus alone has no detectable activity. Rem expression vectors carrying a proline at position 71 show poor cleavage to SP as well as reduced activity in the reporter assay. GFP-tagging of Rem at the N-terminus allows cleavage to GFP-SP, which is active in the reporter assay. However, addition of the GFP tag to the Rem C-terminus shows poor cleavage to SP and little reporter activity. GFP-tagging at the N-terminus also appears to increase stability of SP, but not its specific activity in functional assays. The amount of transfected *rem* expression vector influences cleavage to SP. Transfection of large amounts of expression vector leads to an accumulation of uncleaved Rem and no further increases in reporter activity. Analysis of C-terminal truncation mutants of Rem suggests that removal of the C-terminal 50 amino acids has little effect on SP activity. Removal of 100 or 150 amino acids from the Rem C-terminus dramatically affects stability of the truncated protein and activity in reporter assays. These data suggest that the Rem C-terminus interacts with multiple cellular proteins that influence Rem cleavage and trafficking to the nucleus for binding to MMTV RNA.

MUTATIONS OF BASIC RESIDUES IN THE NUCLEOCAPSID REGION OF HIV-1 GAG CAUSE BUDDING DEFECTS

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HIV-1 release is mediated through the PTAP and LYPXnL motifs, termed Late or L domains, located in the p6 region of Gag. These two motifs recruit the cellular proteins Tsg101 and Alix, respectively, and link Gag to the host ESCRT budding machinery via two different routes. We recently reported a new role for the nucleocapsid region (NC) of Gag in assisting p6 during viral budding although the exact mechanism remains unclear. Here we show that mutations of basic residues in NC caused a pronounced decrease in particle release from 293T cells, even though the NC mutant viruses trafficked normally to cell membranes and retained the ability to bind cellular RNAs. Electron microscopy (EM) analysis of these NC mutants showed a clear accumulation of arrested budding particles at the plasma membrane. We analyzed the NC mutant viruses in physiologically relevant T-cell lines such as CEM and Jurkat and found that they displayed severe budding defects as observed by both biochemical and EM analyses. NC mutants' release defects were strikingly similar quantitatively and qualitatively to those seen with Late-domain mutant viruses. Moreover, release defects caused by mutations in NC were fully alleviated by co-expression of the E3 ubiquitin ligase Nedd4-2s that is known to rescue the release of L-domain mutants. This result suggests that mutations of basic residues in NC interfere with the recruitment of cellular factors that facilitate HIV-1 budding. Taken together our data support a new role for the NC domain of Gag in cooperating with L domain functions essential for HIV-1 budding.

RELATIONSHIP OF HYDROGEN BOND INTERACTIONS OF INHIBITORS IN THE ACTIVE SITE OF HIV-1 INTEGRASE AND LIPINSKI'S RULE OF FIVE FOR DRUG-LIKE PROPERTIES.

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We are using the computational software ICM Pro (MolSoft, LaJolla, CA) to assess the hydrogen bond interactions of inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase with the active site of the enzyme (Protein Data Bank entry 1BIS). Many of these inhibitors have drug-like properties that follow Lipinski's rule of five, that were introduced for predicting oral bioavailability. These rules include: molecular mass less than 500 g/mole, log P (the partition coefficient, defined as the ratio of the concentration of a drug in octanol to its concentration in water) less than 5, and no more than 5 hydrogen bond donors and 10 hydrogen bond acceptors (reviewed in J.B. Ealy and V. Kvarta, J. Chem. Ed. 83:1779, 2006). However, some integrase inhibitors do not follow these rules. Thus, we are addressing two questions: (1) Is there a difference in the number of hydrogen bond interactions of drugs that follow Lipinski's rule of five and those that do not?; and (2) Is there is a difference in the amino acid contacts for the two groups of inhibitors? Additionally, this work demonstrates how in silico approaches can introduce undergraduate students at non-research-intensive campuses to research on the acquired immunodeficiency syndrome (AIDS).

LONG-TERM INHIBITION OF HIV-1 WITH RNAI AGAINST CELLULAR COFACTORS.

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RNAi is a sequence-specific gene silencing mechanism induced by double-stranded RNA and a promising tool for the development of a durable gene therapy against HIV-1. Such RNAi attack usually focuses on target sequences within the HIV-1 RNA genome. Despite potent inhibition, we previously observed the rapid selection of RNAi-resistant HIV-1 variants with a point mutation in the target, even for highly conserved viral target sequences. Viral escape can be blocked by a combinatorial RNAi attack on multiple viral target sequences. Alternatively, RNAi-mediated suppression of the level of cellular proteins that are critically involved in HIV-1 replication may also prevent the evolution of RNAi-escape variants, although this concept has not been tested experimentally.
Many cellular co-factors have been described in literature, e.g. based on large RNAi-screens. We set out to probe the importance of 30 cellular co-factors by producing stable knockdowns in a T cell line and testing for the impact on virus replication. Several co-factors appear to be important for cell physiology, as their knockdown resulted in a loss of cell proliferation. Knockdown of 15 co-factors significantly inhibited virus replication without any apparent cytotoxicity. We focused on three top candidate co-factors for which knockdown results in prolonged inhibition of HIV-1 replication; ALIX, TARBP and ATG16. We specifically tested whether HIV-1 can escape from the restrictions imposed by co-factor knockdown and - despite many attempts - were not able to document any viral escape.

HIV-1 GAG RELEASE REQUIRES THE INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR (IP3R)

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The precursor polyprotein, Gag, of HIV-1 is necessary and sufficient for assembly and release of virus-like particles (VLPs). Previous studies have shown that addition of Ca^{2+} to HIV-infected or Gag-expressing cells increased viral particle release. However, no specific cellular factor has been implicated as mediator of physiologic Ca^{2+} provision. Here, we show that efficient release of assembled HIV-1 Gag requires the inositol 1,4,5-triphosphate receptor (IP3R). IP3R is a transmembrane channel through which Ca^{2+} is released from intracellular stores. Depletion of IP3R by siRNA inhibited release of VLPs and infectious virus. Confocal microscopy of IP3R-depleted cells showed significant reduction of plasma membrane-associated Gag. Immuno-electron microscopy showed that Gag was mostly sequestered inside vacuolar structures. Interference with production of and IP3R binding of the IP3 ligand inhibited VLP release, implying the need for IP3R gating activity. These findings identify IP3R as a novel cellular co-factor in viral particle release. Whether calcium provision arose from IP3R-mediated release of store Ca^{2+} or the subsequent Ca^{2+} influx that is coupled to store emptying was determined from the effect of thapsigargin (TG)-induced store Ca^{2+} release on Gag while the cells were in calcium-free media which effectively nullified Ca^{2+} influx. Both the plasma membrane accumulation of Gag and VLP release were enhanced, indicating that store Ca^{2+} facilitated both events. To identify the determinant in Gag that confers the ability to utilize store Ca^{2+}, the p6 region was tested, since Gag release efficiency maps to Late Domains in p6. Using the pharmacological agent 2-aminoethoxydiphenyl borate (2-APB) to block Ca^{2+} influx, the effect on VLP release of WT Gag and a Gag mutant missing the entire p6 region (Δp6-Gag) was determined. 2-APB had no detectable effect on release of WT Gag VLPs but inhibited release of Δp6-Gag. Disruptive mutations in Late Domain motifs in p6 mapped 2-APB resistance to the PTAP motif. By inference, this finding links utilization of store Ca^{2+} to Gag recruitment of Tsg101, which binds the PTAP motif. It is unlikely that IP3R is an effector of Tsg101 since IP3R depletion did not result in defective Gag processing or loss of viral infectivity, events that characterize Tsg101 depletion. Rather, the results suggest that Tsg101 and IP3R play distinct roles in facilitating Gag release.

MASS SPECTROMETRY-BASED FOOTPRINTING OF LEDGF/P75 INTERACTIONS WITH CHROMATINIZED TEMPLATES

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HIV-1 replication requires integration of the viral cDNA made by reverse transcription into the host chromosome for effective infection and maintaining the infected state in the host. The integration step is catalyzed by the retroviral enzyme integrase (IN). In vivo this process is regulated by multiple viral and cellular cofactors. Among these, lens epithelium derived growth factor (LEDGF/p75) has been identified as the principal cellular cofactor critical for effective HIV-1 integration. LEDGF/p75 has been shown to tether the viral IN protein to chromosomal DNA using a C-terminal integrase binding domain (IBD) and an N-terminal domain ensemble that interacts with chromatin. The N-terminal region of LEDGF/p75 contains a PWWP domain, nuclear localization signal, and dual copies of the AT-hook DNA binding element. While the N-terminal region of LEDGF/p75 is essential for interactions with the chromatin, the molecular mechanism of these interactions is not well understood. We have utilized our mass spectrometry-based protein footprinting approach to examine LEDGF interactions with naked DNA and chromatinized templates (MN). This approach allows comparison of the surface topologies of free protein versus protein-DNA complex using amino acid-specific modifiers and MALDI-ToF MS analysis. LEDGF/p75 surface residues readily modified in the free protein but protected from modification in both LEDGF/p75-DNA and LEDGF/p75-MN complexes are interpreted as direct contacts with DNA. Additional protections detected only in the LEDGF/p75-MN complex will reveal potential protein-protein contacts established between LEDGF/p75 and the chromatinized template. Our results have revealed several Lys residues that are surface exposed in free LEDGF/p75 and are protected from modifications by the bound DNA. Interacting amino acids have been mapped to the N-terminal region of the full-length protein, specifically in the PWWP domain, AT-Hook motifs and CR2. Interestingly, these protections are seen only in the context of the full length protein, while the isolated PWWP domain of LEDGF/p75 failed to interact with DNA. We are currently extending the application of the MS-based footprinting approach to probe LEDGF interactions with the chromatinized templates. The results will define the nature of LEDGF/p75 interactions with chromatin and provide important structural details for LEDGF mediated targeting of HIV integration into the active genes.

HIV-1 RESTRICTION IN THE MUSTELIDAE FAMILY OF CARNIVORES

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A number of cell lines derived from certain Carnivoran species, e.g., domestic dog and cat, do not discernibly restrict HIV-1 between entry and integration. Consistent with this, TRIM5a has ablated restriction function due to a truncated B30.2 domain in felidae and the gene is disrupted in the dog. It may be that Carnivorans in general harbor relatively fewer cellular blocks to replication of primate lentiviruses than do other orders of non-primate placental mammals. However, felids encode APOBEC3 proteins that effectively restrict HIV-1 and generalizing about intrinsic cellular restrictions across the Carnivora in either the Caniformia or Feliformia suborders is not warranted. Lion cells, for example, strongly restrict lentiviruses post-entry by unknown mechanisms.

Species in the large Mustelidae (weasel) family (Caniformia) have received comparatively little analysis but a few fibroblast cell lines apparently lack post-entry restricting activity toward N-MLV and HIV-1. Dependency/restriction factors have not been cloned or characterized. Some of these species are accessible small lab animals used for the study of other viruses, e.g., ortho- and paramyxoviruses.

We are characterizing Mustelidae cells (mink, ferret) for ability to support defined phases of retroviral life cycles. Infection of ferret and mink cell lines with pseudotyped HIV-1 reporter viruses showed that these cells support early life cycle steps comparably to human cells. FIV and EIAV vectors were similarly non-restricted and N- and B-tropic MLV were equivalently infectious. Late, Rev-dependent HIV-1 gene expression and virion production were also comparable between human and Mustelidae cell lines. Infectivities of output HIV-1 virions are being compared now. We have stably expressed human CD4 and CXCR4 in the cell lines using various lentiviral vectors and verified efficient gp120-mediated entry. Preliminary evidence suggests that at least one of the lines supports spreading HIV-1 infection. The studies are also being extended to other cell lines, primary PBMCs, splenocytes, and primary solid organ fibroblasts.

A TRUNCATED ISOFORM OF CPSF6 BINDS TO RETROVIRAL CAPSID PROTEIN AND INHIBITS NUCLEAR ENTRY

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A recent cDNA screen identified a truncated isoform of the nuclear factor CPSF6 that limited the nuclear import of HIV-1 and SIVmac when over-expressed in vitro. A block to nuclear import was implied by a reduction in 2-LTR circle formation. Here we show that over-expression of a related mutant, ΔCPSF6, truncated at a similar position, restricts HIV-1, HIV-2 and SIVmac, but not distantly related lentiviruses FIV and EIAV. Whilst the putative CPSF6 nuclear localisation signal has been lost in these truncations, immunofluoresence staining suggests that the ΔCPSF6 truncation is capable of entering the nucleus. Over-expression in both human and non-primate cells has the same effect on lentiviral infection suggesting that the restriction is virus and not host specific. We use isothermal calorimetry (ITC) to show that a CPSF6 derived peptide binds HIV-1 capsid (CA) and CypA-binding site mutant G89V but not ΔCPSF6 escape mutant N74D. Furthermore, restriction by ΔCPSF6 can be saturated by co-expression of full length HIV-1 CA. Interestingly, mutation of the residue corresponding to N74 in SIVmac and HIV-2 (N73D) also allows their escape from ΔCPSF6. We propose that ΔCPSF6 blocks infection by binding to CA and preventing interaction with the nuclear import machinery.

BIOPHYSICAL ANALYSIS OF HTLV-1 GAG TRAFFICKING AND PARTICLE RELEASE

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The Gag protein is required for virus particle assembly and release. Nonetheless, crucial mechanistic details remain poorly understood, particularly for the deltaretroviruses – the prototype being human T-cell leukemia virus type 1 (HTLV-1). In this study, we investigated Gag stoichiometry in virus particles and as well as Gag-Gag interactions in living cells. We used the novel biophysical technology of fluorescence fluctuation spectroscopy (FFS) in conjunction with cryo-electron microscopy (cryo-EM), to study (1) Gag behavior (FFS) at the single molecule level in living cells, (2) Gag stoichiometry (FFS), and (3) virus-like particle (VLP) size (FFS & Cryo-EM) and morphology (Cryo-EM). FFS determines the brightness, mobility, and concentration of fluorescent particles from the intensity bursts generated by individual particles passing through a small observation volume, which yields information about protein stoichiometry, interactions and transport. Experiments focused in two areas: (1) the self-associative behavior of HTLV-1 and HIV-1 Gag-YFP and various Gag-YFP mutants in the cytoplasm, which can provide insight into the earliest events initiating viral assembly, (2) the stoichiometry and size of HTLV-1 and HIV-1 Gag-YFP complexes present in VLPs, which can provide insight on the possible relation of Gag stoichiometry to virion size. Cytoplasmic studies using FFS revealed that HTLV-1 and HIV-1 Gag exhibit distinct differences in cytoplasmic behavior – such as mobility and oligomerization – that appeared to be dependent on the myristoyl moiety. FFS VLP studies revealed distinct differences in Gag stoichiometry and particle size between HTLV-1 and HIV-1 VLPs. Cryo-EM of HTLV-1 VLPs confirmed the differences in VLP size compared to HIV-1, as well as allowing comparison of HTLV-1 and HIV-1 VLP morphology. In summary, FFS and cryo-EM are complimentary and versatile techniques that can be used to investigate the full range of retroviral Gag behavior, from the initial stages of virus assembly, to the Gag complexes present in VLPs.

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CELLULAR AND VIRAL FACTORS REGULATE THE BUDDING OF FELINE ENDOGENOUS RETROVIRUS

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RD114 virus is a feline endogenous retrovirus and appears to be produced as infectious viruses in some feline cell lines. Recently, we have reported that the isolation of an infectious RD114 virus in a proportion of live attenuated vaccines for pets. It is very difficult to completely exclude the proviral DNA of RD114 from cells, since endogenous retroviruses (ERVs) are usually integrated in the multi-locus of host chromosome. However, it may be possible to reduce the risk by regulating the release of infectious RD114 from cells. For this purpose, in this study, we analyzed the molecular mechanism of RD114 virus budding and examined the antiviral activity of Tetherin/BST2 for RD114. The analysis for L-domain mutants of RD114 showed that the PPPY sequence play a critical role in RD114 release as viral L-domain. Furthermore, we investigated the cellular factors required for RD114 budding. We demonstrated that RD114 virus release was inhibited by overexpression of dominant negative mutants of Vps4A, Vps4B, and BUL1, but not Nedd4.1, Tsg101, and Alix. These results strongly suggest that RD114 budding utilizes the cellular multivesicular body (MVB) sorting pathway as well as other many retroviruses. In addition, we observed that human Tetherin/BST2 significantly inhibited the production of RD114 from cells. We are also investigating the anti-RD114 activity of feline Tetherin/BST2. These data would be useful for development of strategies to inhibit RD114 production in feline cells.

THE K65 AND K66 RESIDUES OF HIV-1 REVERSE TRANSCRIPTASE ARE DETERMINANTS OF PRIMER MISPAIR EXTENSION FIDELITY

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HIV-1 reverse transcriptase (RT) is responsible for replicating the HIV genome, and the errors during this replication result in the genetic variation from which drug- and immune escape variant viruses emerge. In order for a point mutation to occur, a nucleotide must be misinserted, and the mismatched primer terminus thus formed must be extended further. HIV-1 RT is relatively efficient at both misinsertion and mismatched primer extension. While the influence of K65 and other residues in RT on misinsertion fidelity has been previously examined, factors influencing mispair extension are poorly understood. We have examined the role of K65 and K66, in the fingers subdomain of RT in these two aspects of polymerase fidelity. While K65 is conserved among nearly all retroviruses, K66 is conserved among lentiviruses and betaretrovirus RTs.

We previously hypothesized that K65 enhances misinsertions via interactions with the γ-phosphate of incoming dNTP. Here, we present pre-steady state kinetic analysis of the wild type HIV-1 RT and two substitution mutants, K65A and K65R, which supports our hypothesis. Next, we examined extension by all three RTs on matched and mismatched primer termini. All enzymes discriminated against the mismatched primer at the catalytic step (k_{pol}). Both K65A and K65R mutants displayed a further decrease in mismatch extension efficiency, primarily at the level of dNTP binding. Our results show the influence of the stabilizing interactions of Lys65 with the incoming dNTP in affecting two different aspects of polymerase fidelity.

The K66 residue is positioned close to the penultimate base-pair of the primer strand; we hypothesized that this residue could also influence the efficiency of extension from mismatched primer termini. We tested this hypothesis using wild-type HIV-1 RT and four substitution mutants: K66R/A/N/T. All K66 substitution mutants were inefficient at mispair extension, showing up to a 10-fold difference compared to wild-type, suggesting that Lys66 is a determinant of mispair extension.

Using site-specific hydroxy radical footprinting, we demonstrated that the wild type HIV-1 RT occupies a non-productive position (-2 with respect to the templating base) on the mismatched primer, compared to position +1 on matched template-primer. The lysine substitutions at either the K65 or K66 did not alter this change in position when presented with the mismatched primer-template, but rather reduced nucleotide utilization in the context of the mismatched terminus. Our results suggest that HIIV RT has evolved mechanisms to decrease overall fidelity, and that the mechanisms for misinsertion and mismatch primer extension act in concert.

TRANSCRIPTIONAL AND TRANSLATIONAL ANALYSES OF THE CELLULAR RESPONSES INDUCED BY THE INTEGRATION OF HIV INTO TARGET CELLS.

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The replication cycle of retroviruses requires the integration of the viral genome into the genomic DNA of the infected cells. The aim of our work is to identify cell signalling pathways that might be specifically activated or repressed during Human Immunodeficiency Virus (HIV) integration to maintain genome integrity. We infected human fibroblast cells with an HIV-based lentiviral vector devoid of a reporter gene. As a control, we used the same vector that was defective for integration but that still remained able to perform the early steps of integration (i.e. entry, reverse transcription, PIC transport…). By nested quantitative PCR assays, (i) we determined the kinetic of integration of the first vector at different multiplicity of infection (ii) we checked that the second vector was unable to integrate. Then, at a time post-infection corresponding to the beginning of the exponential curve of integration, we took RNAs and proteins of cells infected with one or the other vectors. RNAs were studied using human microarrays (U133 Plus Whole Human Genome, Affymetrix). Proteins were analyzed on 2D denaturing gels. By comparing RNAs and proteins from each cell population, we were able to detect and discriminate signals specifically due to integration (Ingenuity Pathways Analysis Software, Ingenuity systems). These analyses led us to identify several major cellular processes in which RNA and proteins are up- or down- regulated. These processes include DNA repair, innate immunity, chromatin remodelling. We selected 20 factors for their biological relevance. We are currently analysing their expression, in cells infected with one or the other vectors, by RT-qPCR or Western Blot. As the integration of additional DNA not only concerns viral processes but also cellular processes (transposition of mobile elements, chromosomal reorganizations during cancers induction..), data obtained in this study may allow: (i) the development of defensive strategies against integrative viruses (such as HIV (ii) the evaluation of cellular perturbations caused by the integration of a therapeutic vector into the cell genomes during gene therapy or gene transfer experiments

FORMATION OF CHROMATIN-ASSOCIATED NUCLEAR FOCI CONTAINING HIV-1 VPR AND VPRBP IS CRITICAL FOR THE INDUCTION OF G2 CELL CYCLE ARREST

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HIV-1 Viral protein R (Vpr) induces a cell cycle arrest at the G2/M phase by activating the ATR DNA damage/stress checkpoint. Recently, we and several other groups showed that Vpr performs this activity by recruiting the DDB1-CUL4A (VPRBP) E3 ubiquitin ligase. While recruitment of this E3 ubiquitin ligase complex has been shown to be required for G2 arrest, the subcellular compartment where this complex forms and functionally acts is unknown.
Using immunofluorescence and confocal microscopy, we show that Vpr forms nuclear foci in several cell types including HeLa cells and primary CD4+ T-lymphocytes. These nuclear foci were found to co-localize with VPRBP and the DNA repair factors 53BP1 and RPA32. While treatment with the non-specific ATR inhibitor caffeine or depletion of VPRBP by siRNA did not inhibit formation of these foci, mutations in the C-terminal domain of Vpr and cytoplasmic sequestration of Vpr by overexpression of Gag-Pol resulted in impaired formation of these nuclear foci. Consistently, we observed that G2 arrest-competent sooty mangabey Vpr could form these foci but not its G2 arrest-defective paralog Vpx, suggesting that formation of these foci represents a critical early event in the induction of G2 arrest. The inability of Vpr C-terminal mutants to form foci did not result from inefficient dimerization, as measured by bioluminescence resonance energy transfer (BRET), but rather correlated with defective chromatin association. Indeed, nuclease treatment of cellular extracts showed that Vpr could associate to chromatin via its C-terminal domain and that it could form a complex with VPRBP on chromatin.
Overall, our results suggest that Vpr would target a chromatin-bound cellular substrate for ubiquitination in order to induce DNA damage/replication stress, ultimately leading to ATR activation and G2 cell cycle arrest.

THE ROLE OF LEDGF/P75 CHROMATIN BINDING DOMAINS IN HIV INTEGRATION SITE TARGETING.

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Proviral integration of HIV and HIV-derived lentiviral vectors favors transcriptionally active regions. LEDGF/p75, a transcriptional co-activator, is the dominant cellular co-factor of HIV-1 integrase (IN) that tethers the PIC to chromatin. IN interacts with the IN binding domain (IBD, aa 347-429) in the C-terminus of LEDGF/p75. Stable overexpression of the LEDGF/p75 C-terminal end ($LEDGF_{325-530}$) results in a severe inhibition of HIV replication. LEDGF/p75 coordinates the nuclear accumulation and chromosomal localization of HIV IN via an N-terminal domain containing an ensemble of chromatin binding elements, containing a PWWP-domain (aa 1-93) and two AT-hook-like domains; however, the exact orchestration of this process remains unknown.
In order to study the role of the LEDGF PWWP domain, we generated a series of PWWP-truncation mutants (ΔN18, ΔN26, ΔN30, ΔN50, ΔN85, ΔN93) and chimeric proteins, replacing the PWWP-domain of LEDGF/p75 with that of HDGF and HRP-2. Together with LEDGF/p75, HDGF and HRP-2 are members of the HRP-family sharing a conserved PWWP domain. Next to LEDGF/p75, HRP-2 is the only other protein that contains an IBD-like domain in addition to a PWWP-domain. All constructs were stably expressed in LEDGF/p75-deficient cells. All mutant proteins migrated at the expected size in Western analysis and located to the nucleus. All cell lines were tested for lentiviral vector transduction and HIV-1 infection (HIV ΔNefΔEnv-fLuc or HIV NL4.3).
In our hands, PWWP-truncations do not rescue viral vector transduction or HIV infection in LEDGF deficient cells. Swapping the PWWP-domain of LEDGF with that of either HDGF or HRP-2 rescued LV transduction and HIV replication to nearly wild-type levels. Also, introduction of HRP-2 in LEDGF-depleted cells rescued HIV replication. Analysis of proviral integration sites revealed an integration site pattern for HRP-2 that is not different from LEDGF-depleted cells, whereas the integration site profile for the PWWP-swaps is similar to that of WT LEDGF.

MECHANISM OF RECEPTOR INDEPENDENT ENTRY OF ALVs WITH EXTENDED HOST RANGE.

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Avian leukosis viruses (ALVs) are the type members of the *Alpharetrovirus* genus, and are classified into ten subgroups (A-J) distinguished by receptor usage, host range, and superinfection resistance patterns. Subgroups A-F display remarkable receptor usage variation despite having very similar env sequence(approximately 80% identity). The receptor-binding specificity of ALV has been mapped to two regions within SU, hr1, and hr2, by our laboratory. We have studied the evolution of receptor usage in avian retroviruses based on the hypothesis that all 10 subgroups evolved from a common ancestor through accumulation of point mutations within these regions. Previously, we have described two independent mutations in hr1 of subgroup B ALV that allow infection of receptor-negative cells. We found that introduction of these mutations into subgroup C, but not A virus, conferred a similar host range extension (HRE) phenotype. We extended the analysis to show that many, but not all, additional mutations tested at these sites, produced a similar HRE phenotype in subgroups B and C. Here we show that subgroup C HRE mutant L154S is able to infect cells in a receptor independent manner. In support of this conclusion, we found that the ALVC L154S virus was less sensitive to superinfection resistance compared to the wild-type (WT) ALVC virus and that binding assays showed that the L154S mutation does not result in the binding of an SU immunoadhesin to a new receptor on receptor negative cells. Additionally, the ALVC L154S virus was inactivated by pre-incubation with low pH buffer, while the ALVC WT virus was not, suggesting the loss of the requirement for receptor-induced conformational change for low pH mediated entry of WT virus. Peptide R99, which inhibits the formation of the six helix bundle (6HB) of ALV, inhibited the infection of receptor positive cells by ALVC WT and L154S. We are currently extending this analysis to the additional mutants we have previously described for subgroups B and C to determine if all use a similar mechanism. Taken together, our data support a model in which these mutants represent evolutionary intermediates that allow the virus to respond to selective pressure resulting from the absence of functional receptor in the host and subsequently to adapt to use new receptors for entry.

VPX COUNTERACTS A BLOCK TO RETROVIRUS INFECTION BUT DOES NOT PROMOTE MLV INFECTION OF MACROPHAGES

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Lentiviruses of the HIV-2/SIVmac/SIVsm lineage encode the non-structural protein, Vpx. Vpx facilitates the infection of macrophages and dendritic cells where it has been proposed to neutralize the antiviral activity of an unidentified restriction factor. In monocyte derived macrophages (MDM) infected with *Δvpx* SIVmac, the production of early, late and 2-LTR circular viral cDNA was markedly reduced, suggesting that replication was blocked prior to nuclear import and possibly during uncoating. The defect could be restored by pre-incubation of MDM with virus-like particles (VLP) loaded with Vpx. Vpx-containing VLP also potently increased the infectivity of HIV-1 on MDM, although this virus does not encode for Vpx. To test whether the effect of Vpx is specific to SIV and HIV or whether other retroviruses are affected by this block, we infected MDM with FIV, EIAV, N-, B-, and NB-tropic MLV GFP reporter virions in the presence or absence of Vpx-containing VLP. By analyzing single-cycle infections using flow cytometry, we found that Vpx-VLP enhance infection of FIV and EIAV, but do not promote MLV infection of MDM. MDM from some donors showed low level of infection for B- and NB-tropic MLV but no significant impact of Vpx on infectivity could be observed. Infection of MDM by N-tropic MLV with or without Vpx was low, probably due to the additional restriction by human TRIM5alpha. We measured the accumulation of NB-tropic MLV cDNA at different time points post-infection by qPCR. We found that Vpx enhanced the formation of early and late reverse transcripts indicating that Vpx overcomes a block early in MLV infection, similar to its effect on HIV and SIV. However, Vpx failed to enhance infection beyond that step, probably due to impaired nuclear import of MLV in non-dividing MDM. Taken together, our results show that Vpx counteracts a cellular restriction that affects not only SIV and HIV, but a variety of retroviruses including FIV, EIAV and MLV at the level of reverse transcription. However, preincubation of MDM with Vpx-containing VLP was not sufficient to lift the block to MLV infection in MDM.

INVESTIGATION OF XMRV AS A HUMAN PATHOGEN

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Xenotropic murine leukaemia virus-related virus (XMRV) has recently been associated with prostate cancer and more controversially with chronic fatigue syndrome in humans. It has also been claimed that XMRV is present in a low percentage of apparently healthy individuals. Phylogenetic analysis reveals that XMRV is a gammaretrovirus closely related (92% similarity) to endogenous xenotropic murine gamma retroviruses that are often found as contaminants in laboratory cell lines. Here we have screened 411 human tumour cell lines by Taqman QPCR and found that 9 (2.2%) are infected with xenotropic murine leukaemia viruses. Further, neither XMRV, nor related sequences, were detected in blood samples from 543 HIV-1 infected individuals. XMRV has been described as having a unique deletion of 24 nucleotides in the gag leader sequence allowing its specific detection by PCR. We applied phylogenetic methods to determine the origin of XMRV amongst endogenous gamma retroviruses found in inbred mice, but failed to identify an unambiguous ancestor. However, we have found evidence for sequences described as XMRV-specific in at least one inbred mouse strain.

EFFECTS OF FACTORS MODULATING TRANSLATIONAL READTHROUGH OF THE GAG-POL STOP CODON ON RETROVIRAL REPLICATION

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The production of retroviral Pol enzymes in the gammaretroviruses is controlled by translational readthrough of the stop codon at the end of the gag ORF by secondary mRNA structures – pseudoknots -- in the pol coding sequence. Pol gene products are made only as a fusion product with the upstream Gag proteins; translation of pol genes depends on suppression of the normal termination event. This occurs via incorporation of an amino acid at a UAG stop codon and continued translation. Gag-pol products are produced in approximately 5% of ribosomal elongation cycles, and close adherence to the normal gag:gag-pol ratio is necessary for efficient viral replication. We have previously shown that large ribosomal protein L4 (RPL4) increases readthrough at the MoMLV/XMRV gag:gag-pol junction (containing a well-defined pseudoknot structure) in a dose-dependent manner in a dual-luciferase reporter context. We are now investigating the effects of aminoglycoside antibiotics and small molecules thought to suppress premature stop codons on the retroviral readthrough event. Preliminary results show that these molecules increase readthrough at the MoMLV/XMRV pseudoknot in our reporter assays. We are also analyzing the effect of RPL4 and readthrough promoting drugs on viral replication: specifically, we are asking whether increasing readthrough diminishes the ability of these viruses to replicate. Effectiveness of particular molecules with well-characterized modes of function may elucidate how the MoMLV/XMRV pseudoknot interacts with the translation machinery to promote readthrough.

CONTRIBUTION OF THE MLV AND HIV-1 MA DOMAINS TO SPECIFIC ENV RECRUITMENT.

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The incorporation of functional Env glycoproteins is a necessary aspect of assembling an infectious virion. As well as incorporating native Env, retroviruses are also able to incorporate some foreign glycoproteins, a process known as pseudotyping. Though it is known to be an active process, the mechanism for incorporation still remains largely a mystery. Several studies have provided evidence for direct and indirect interactions between HIV-1 matrix (MA) and HIV Env. It is likely that such interactions are responsible for native Env incorporation for all retroviruses. For pseudotyping, the recruitment of foreign glycoproteins, MA to Env interactions are still possible, though less likely. To help elucidate the mechanisms of recruitment for both native and foreign Env proteins, we made HIV-1 and MLV constructs with the MA domain replaced by either non-viral membrane binding domains or the MA domain of a foreign retrovirus. These constructs were assayed for the ability to recruit and form infectious particles with various viral glycoproteins. Our findings suggest that HIV MA is both necessary and sufficient for HIV-1 Env recruitment and infectivity. Replacing HIV MA with any other domain significantly reduced infectivity. Conversely, although MLV is poorly infectious when pseudotyped with HIV-1 Env, replacement of MLV MA with HIV MA greatly enhances this infectivity. Unlike HIV Env, MLV Env is a promiscuous glycoprotein that can efficiently form infectious particles with HIV-1 or MLV. However, if MLV and HIV are introduced into the same cells and forced to compete for limiting Env, MLV Env strongly favors the formation of infectious MLV particles. Replacement of MLV MA with HIV MA reduces, but does not eliminate this selectivity. The selectivity shown by MLV is MLV Env specific and requires only Gag, though Pol also appears to contribute.

POSSIBLE RECRUITMENT OF TETHERIN TO ASSEMBLING HIV-1 VIRIONS THROUGH PLASMA MEMBRANE MICRODOMAINS

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Tetherin is a cellular restriction factor that inhibits the release of enveloped viruses from host cells. Recent evidence suggests that this restriction occurs via physical tethering of host and viral membranes, followed by endocytosis of tethered virions. Tetherin has been shown to co-localize with HIV-1 Gag protein by immunofluorescence microscopy and with plasma-membrane-tethered virions by immuno-TEM. The specific mechanism of this co-localization is currently unknown. Given tetherin's ability to restrict diverse enveloped viruses, it is unlikely that tetherin is recruited to assembling virions by binding to specific viral proteins. We hypothesized that this recruitment may instead be facilitated by a common feature of many enveloped viruses such as plasma-membrane microdomains.

Tetherin contains two membrane-interacting domains: its transmembrane domain and C-terminal GPI anchor. These two domains, along with its dimerization capability, are thought to facilitate simultaneous incorporation of tetherin into cell and viral membranes. Tetherin, like other GPI-anchored proteins, is thought to reside in cholesterol-enriched microdomains known as lipid rafts, based on the presence of tetherin in detergent-resistant membrane fractions. We and others have shown the likely involvement of lipid rafts in HIV-1 assembly, providing a possible mechanism for the recruitment of tetherin to assembling virions. In addition, it has been reported that tetraspanin-enriched microdomains (TEMs) also associate with sites of virus assembly.

To determine whether tetherin associates with lipid rafts or TEMs, copatching of tetherin with antibody-crosslinked specific microdomain markers was examined by fluorescence microscopy. Preliminary data show that endogenous tetherin in HeLa cells does co-localize with a lipid raft marker, CD59, but not with tetraspanins CD9, CD63, and CD81 in the absence of Gag.

To further examine the relationship between tetherin and assembling virions, we utilized HIV-1 Gag mutants with different membrane binding properties. In these studies WT Gag is compared to a mutant with the triple-acylation signal of Fyn kinase in place of the N-terminal myristate, which is capable of cholesterol-independent assembly. Surprisingly, our data suggest that both forms of Gag are susceptible to tetherin restriction in both untreated and cholesterol-depleted cells. These results suggest that microdomain association of tetherin alone fails to completely account for its specific recruitment to assembling virus particles.

ALANINE SCANNING MUTAGENESIS OF THE GP120 GLYCOPROTEIN AND THE IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED IN CXCR4 SIGNALING

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Previously we have demonstrated that HIV-1 gp120-CXCR4 interaction triggers signal transduction critical for the latent infection of human resting CD4 T cells. This study served to identify amino acid residues of the gp120 glycoprotein responsible for mediating signal transduction in human resting CD4 T cells. The env gene of HIV-1_{NL4-3} was cloned into a vector and subjected to alanine scanning mutagenesis. A subset of 22 amino acid residues occurring in the V1/V2 loop, V3 loop, β19 sheet, C1 and C2 domains were selectively mutated and used to produce viral particles expressing mutant gp120 glycoproteins. Resting CD4 T cells were infected with the viral mutants and analyzed for the activation of signaling molecules such as cofilin. Cytoskeletal actin rearrangement mediated by the gp120 mutants were also measured with F-actin staining and flow cytometry. In addition, the impacts of the gp120 mutations on the ability of HIV-1 to establish latent infection of resting CD4 T cells were further evaluated.

THE SIGNIFICANCE OF DUPLICATE READS IN HIGH THROUGHPUT SEQUENCING OF INTEGRATION SITES

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High throughput sequencing such as Roche 454 and Illumina are powerful tools for investigating the integration patterns of transposons and retroviruses. They usually provide tens of thousands of integration positions in one sequencing reaction. When blasting the sequences of integration sites we often find many sequence reads are duplicated, suggesting multiple integration events occurred at the same chromosome coordinate and in the same strand. There could be three origins for duplicate reads. They could result from independent integrations, the growth of cells with integration, or, PCR amplification. Since it is hard to know what fraction of the duplicate reads represent independent events, researchers avoid analyzing the duplicate sequences. We find studying the duplicate sequence reads does help us in understanding the integration profile of target sites.

In a previous study, we used 454 sequencing to study the integration of retrotransposon Tf1 in *Schizosaccharomyces pombe*. We sequenced approximately 20,000 insertions in each of four independent experiments and obtained a saturated collection of target sites. Here we report the insertions have a distinct preference for specific nucleotides that extends 50 base pairs on either side of its insertion sites. We find the nucleotide preferences are enhanced substantially if the duplicate sequence reads are included. We also find that Tf1 integrates where nucleosome occupancy is relatively low. Interestingly, Tf1 integration sites with higher numbers of duplicate reads tend to have lower nucleosome occupancy. These observations indicate that at least some of the duplicate reads are from independent insertion events. Therefore, the duplicate reads can reveal important information about the targeting frequency of integration sites. When we deleted the chromodomain from the integrase of Tf1, the positions targeted by the transposon correlated well with those targeted by wild type Tf1. However, when the duplicate reads are considered, we find the mutant transposon has a substantial change in the integration frequency of the target sites. This indicates that if the duplicate reads are discarded, low frequency target sites can not be distinguished from the highly active sites. In order to distinguish independent insertion events from the results of cell duplication and PCR amplification, we have designed a version of Tf1 with a "serial number" tag system. Thus, we will be able to measure the targeting frequency of individual nucleotide positions.

ORIGINS AND EVOLUTIONARY BIOLOGY OF HIV-1 GROUP P

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A single novel HIV-1 strain RBF168, related to simian immunodeficiency virus from gorillas (SIVgor), has been designated group P. It was isolated from a woman of Cameroonian origin with no history of exposure to bushmeat. Here we have conducted a phylogenetic and in vitro study into RBF168 in order understand its evolutionary history. After extensive analyses we found no evidence to support a recombinant origin for this virus. We also dated the HIV-1 group P and SIVgor most recent common ancestor (tMRCA) using a Bayesian framework, as an estimate of the time at which the two lineages diverged from each other. We found good agreement in the RBF168/SIVgor tMRCA estimates obtained on the basis of the gag (1899 [95% Higher Probability Density: 1855-1938]) and env genes (1845 [1766-1912]). RBF168 also showed a lack of adaptation to humans in lineage specific positive selection analyses on the gag, pol, env, vpu and nef genes. However, there was evidence for adaptation in the vpu gene in branches leading to HIV-1 group M and N as well as in the branch leading to the SIVgor/HIV-1 group P/HIV-1 group O clade (corresponding to SIV zoonotic events). Of note, no evidence for adaptation was found in the nef phylogeny for any branches. Like group O, neither HIV-1 group P Vpu or Nef proteins were able to antagonise restriction by human tetherin. Furthermore, although SIVgor Nef was able to counteract gorilla tetherin, RBF168 Nef could not. This suggests that RBF168 is not adapted to replicate/transmit well in humans or gorillas. Overall our data suggest that despite diverging from SIVgor about 150 years ago, group P represents a rare zoonotic virus that has failed to adapt well to its new human host leading to low transmission rates and low incidence.

THE XENOTROPIC MURINE LEUKEMIA VIRUS-RELATED VIRUS IS INHIBITED BY APOBEC3 PROTEINS AND ANTI-HIV-1 DRUGS

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Recent studies have reported the first gammaretrovirus that infects humans, named xenotropic murine leukemia virus-related virus (XMRV). XMRV was isolated from human prostate cancer tissue and from activated $CD4^+$ T cells and B cells of patients with chronic fatigue syndrome, suggesting an association between XMRV infection and these two disease states.

Since APOBEC3G (A3G) and APOBEC3F (A3F), which are potent inhibitors of the murine leukemia virus and Vif-deficient human immunodeficiency virus type 1 (HIV-1), are expressed in human $CD4^+$ T cells and B cells, we sought to determine whether and how XMRV evades suppression of replication by APOBEC proteins. Surprisingly, we found that XMRV infectivity is potently inhibited by A3G and A3F, and to a lesser extent, by murine APOBEC3 expressed in viral producer cells. All three APOBEC3 proteins were packaged in XMRV virions and induced G-to-A hypermutation of the proviral DNA. Analysis of A3G and A3F mRNA levels in different cell lines using quantitative real-time RT PCR indicated that human T-cell lines CEM and H9 exhibited higher A3F mRNA levels than prostate cell lines 22Rv1, LNCaP, and DU145. In contrast to high levels of A3G mRNA in CEM and H9 cells, A3G mRNA was not detectable in 22Rv1 and LNCaP cells and very low in DU145 cells. We found few XMRV proviral genomes with G-to-A hypermutation in 22Rv1, LNCaP and DU145 cells, primarily in the GA dinucleotide context, indicating A3F activity. In A3G/A3F-positive T cell lines (CEM and H9) XMRV proviral genomes were extensively G-to-A hypermutated, but not in CEM-SS cells with low A3G/A3F expression. These hypermutations occurred mostly in a GG dinucleotide context known to be targeted by A3G and caused stop codons in nearly all analyzed proviruses.

We also analyzed the sensitivity of XMRV to several antiretroviral drugs and compared the 50% inhibitory concentration values (IC_{50}) to those for HIV-1 inhibition. XMRV was found to be susceptible to the nucleoside reverse transcriptase inhibitors AZT and tenofovir and the integrase inhibitor raltegravir at similar concentrations to those that inhibit HIV-1.

Overall, these results show that XMRV is targeted and inhibited by the tested APOBEC3 proteins. Therefore, cells that express low levels of A3G/A3F, such as prostate cells, may provide a suitable target for XMRV replication and spread. Furthermore, anti-HIV-1 drugs AZT, tenofovir, and raltegravir may be useful for treatment of XMRV infection.

ROLE OF HIV-1 VIF POLYUBIQUITYLATION IN APOBEC3G PROTEASOMAL DEGRADATION

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The major function of primate lentiviral protein Vif is to neutralize the antiretroviral activity of host cytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F). Vif serves as an adaptor protein that bridges A3G/A3F to a Cullin5 (Cul5) E3 ligase. This interaction then triggers the polyubiquitylation of A3G/A3F on lysine residues, resulting in A3G/A3F degradation by proteasomes. However, Vif itself not only has multiple lysines, but also is polyubiquitylated by the same Cul5 E3 ligase. Currently, it is still unclear which polyubiquitylation, either in A3G or Vif, is more critical for A3G proteasomal degradation. Here, we present evidence that Vif polyubiquitylation is required for A3G degradation.

Vif from NL4-3 has 192 amino acids, including sixteen lysines (K22, K26, K34, K36, K50, K63, K91, K92, K141, K155, K157, K160, K168, K176, K179, and K181). Previously, when we mutated all these lysines to arginines (Vif 16K/R), Vif failed to neutralize A3G, although it still bound to Cul5. Because residues K22 and K26 belong to a functional motif 21WxSLVK26, which critically regulates A3G neutralizing activity, we first repaired these two lysines in the Vif 16K/R mutant, and determined its A3G neutralizing activity. However, this new mutant was still completely inactive in A3G neutralization. Since the other lysines are not involved in Vif interactions with A3G, Cul5, and elongin C, this result indicated that additional lysines on Vif are required for A3G neutralization; and most likely, these lysines are involved in Vif polyubiquitylation. Indeed, we were able to map these additional lysines and found that either the N-terminal four lysines (K34, K36, K50, K63) or C-terminal four lysines (K168, K176, K179, K181) could restore a full activity of Vif. Thus, these results not only demonstrate that multiple lysines are required, but also suggest that Vif may have two polyubiquitylation signals, which critically regulate its A3G neutralizing activity.

A NOVEL ROLE FOR APOBEC3A IN FOREIGN DNA RESTRICTION

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Several viruses such as HIV-1, PFV, and SIV are potently restricted by members of the APOBEC3 family of DNA cytidine deaminases. The restriction induced by APOBEC3 proteins is of such consequence to these parasitic elements that they have evolved extensive counter-defense mechanisms. The interaction between the human APOBEC3G protein and HIV-1 Vif is the prototypic example.

APOBEC3A is an understudied member of the APOBEC3 family, possibly because it is such a potent DNA deaminase (frequently toxic to E. coli). In contrast to APOBEC3G, over-expressed APOBEC3A is not efficiently encapsidated in HIV-1 virions. APOBEC3A does not diminish HIV-1 infectivity when it is expressed in producer or target cells. HIV-1 Vif also fails to trigger the degradation of APOBEC3A. These observations strongly suggest that APOBEC3A poses little threat to HIV-1.

However, we recently found that, unlike APOBEC3G, APOBEC3A does have potent activity against foreign DNA substrates such as plasmids and bacterial chromosomal DNA (1). This deaminase-dependent restriction activity results in the hypermutation and degradation of plasmid DNA, greatly limiting procedures such as transient or stable gene transfections. APOBEC3A appears to be expressed exclusively in myeloid lineage cells and its expression is massively upregulated by treating cells with IFNalpha or immunostimulatory DNA. We conclude that APOBEC3A primarily functions as an inducible foreign DNA restriction system analogous to bacterial restriction-modification systems.

Selected Reference:
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ENDOGENOUS HUMAN APOBEC3B INHIBITS HIV-1 REPLICATION

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The seven distinct APOBEC3 cytidine deaminases (A3A-A3H) are part of the human intrinsic immunity against retroviruses. A3B, contrary to A3G/A3F, resists HIV-1 Vif mediated proteosomal degradation. Genome-wide copy number variation studies have shown that the A3 locus is polymorphic among different human populations with one specific copy number variant deleting the complete coding region of A3B. Recently, a large epidemiological study showed a significant association between homozygous A3B copy number loss and increased risk of HIV-1 acquisition and disease progression. We hypothesized, therefore, that human A3B has a profound impact on replication of HIV-1 variants with functional Vif alleles.

In this study, we used lymphoblastoid B cell-lines (LCLs) from HapMap participants because of their physiological A3 expression levels and because of the availability of extensive information on their genomic profiles. LCLs homozygous for the A3B deletion and their matched controls with two A3B copies were used for infection experiments using wild-type and VSV-G pseudotyped HIV-1 (n=18). Endogenous A3B mRNA expression was measured using qRT-PCR. We could not detect any A3B mRNA in the LCLs homozygous for the A3B deletion whereas the expression varied considerably (>20 fold) between the LCLs with two A3B copies. We found that LCLs homozygous for the A3B deletion were significantly more susceptible to HIV-1 infection than wild-type controls (Mann-Whitney U test: $p<0.05$). The restriction exerted by A3B was independent of viral entry. Transduction of LCLs harboring two A3B copies with shRNA directed against A3B decreased A3B mRNA by 80% and strongly increased HIV-1 replication (100-fold, replication competent full-length, Vif wild-type NL4-3, MOI: 0.005).

Our results demonstrate that the Vif-resistant A3B is expressed in LCLs and efficiently inhibits HIV-1 replication. Conversely, knocking down A3B expression increases HIV-1 replication. Taken together, A3B might play a critical role in the host intrinsic defense against HIV-1, in a cell type specific manner. Consequently, individuals that have a homozygous loss of this gene may have an increased susceptibility to HIV-1 infection. This observation has important public health consequences considering the high frequency (>10%) of the A3B deletion among Asian populations.

HOMOLOGOUS LOOPS CONTRIBUTE TO THE DISTINCT ANTIVIRAL ACTIVITIES OF HUMAN APOBEC3A AND APOBEC3G

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Cytidine deaminases of the APOBEC3 family are editing enzymes endowed with restriction activity against a variety of mobile genetic elements. Human APOBEC3A can block efficiently the parvovirus adeno-associated virus type 2 (AAV-2) or the retrotransposon LINE-1 but cannot inhibit human-immunodeficiency virus-1 (HIV-1) replication, while APOBEC3G can block HIV-1 but not AAV-2 or LINE-1. The molecular determinants underlying the susceptibility of each element to one or the other APOBEC3 proteins have remained largely undefined or controversial. Here, we performed a comprehensive functional analysis of evolutionarily conserved residues in human APOBEC3A and APOBEC3G required for inhibition of two viruses, HIV-1 and AAV-2, and two retrotransposons, LINE-1 and Alu. Despite different intrinsic activities of the two proteins, structural modeling revealed that functionally important residues mapped on homologous loops close to the active site. Phylogenetic analysis showed that the nature of the loops largely differed between APOBEC3A and APOBEC3G primate paralogues including at amino-acids of functional importance, in stark contrast with secondary structure elements. The residues important for restriction activity mediated intersubunit contacts between APOBEC3G monomers and the predicted dimer interface shaped a groove that can accomodate single-stranded nucleic-acids, in agreement with experimental data. For APOBEC3A however, it is on the monomer that residues critical for restriction and editing activities formed a groove with single-stranded DNA binding potential. In summary, this study unveils critical determinants of restriction activity that mapped on homologous loops between APOBEC3A and APOBEC3G, and suggests that target selectivity by APOBEC3 proteins may stem from structural variations in this region.

PERTURBATION OF THE P-BODY COMPONENT MOV10 INHIBITS HIV-1 INFECTIVITY

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Here we demonstrate that Mov10, a protein associated with P-bodies that has a putative RNA-helicase domain, can inhibit the production of infectious retroviruses when ectopically expressed. Although Mov10 co-purifies with cytoplasmic APOBEC3G, the inhibition appears to be APOBEC3G-independent as Vif-positive HIV is Mov10-sensitive, and Mov10 impairs HIV production from APOBEC3G-negative cells. At intermediate levels of Mov10 overexpression, the specific infectivity of HIV is impaired, whereas increased levels of Mov10 potently inhibit both the specific infectivity and production of HIV. HIV expressed in the presence of high levels Mov10 exhibits diminished amounts of processed Gag products, and virions that are released display maturation defects. Mov10 directly interacts with HIV. It associates with HIV RNA during virus production and is subsequently incorporated into viral particles. Interestingly, reducing endogenous Mov10 levels in virus-producing cells through siRNA treatment also modestly suppresses HIV infectivity suggesting a positive role in infection. The actions of Mov10 are not limited to HIV, as ectopic expression of Mov10 restricts the production of other lentiviruses as well as the gammaretrovirus, murine leukemia virus. We mutated domains of Mov10 to better understand its inhibition of HIV when overexpressed. While specific mutation of the Mov10 RNA helicase domain hindered virion incorporation, it did not eliminate antiviral activity. Similarly, the N-terminal domain of Mov10, which is not incorporated into virion particles, was also sufficient to impair both the production and specific infectivity of HIV. Together these results indicate that manipulation of Mov10 levels may provide a potent antiviral strategy. These data also suggest a link between cytoplasmic RNA machinery and infectious retrovirus production.

MOLONEY LEUKEMIA VIRUS 10 PROTEIN INHIBITS RETROVIRUS REPLICATION

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The MOV10 gene locus was first discovered in M-MuLV-infected mice when the provirus was inactivated after integration into this site. It encodes a 110 kDa GTP-binding protein, and was classified as a SF-1 RNA helicase. The function of MOV10 remained unknown until its orthologs SDE3 in Arabidopsis and Armitage in Drosophila were found to play a critical role in the RNAi pathway. SDE3 was isolated as the first non-RNA-dependent RNA polymerase (RdRP) factor in plants that amplifies RNAi by increasing the production of dsRNA, whereas Armitage was found to be required for RISC assembly. The role of MOV10 in RNAi was further highlighted in human. It was found that MOV10 not only interacts with Ago2, but MOV10 also is associated with a multiprotein complex containing TRBP and eIF6, which are another two important components of RISC. A depletion of MOV10 from cells seriously compromised the activity of the RNAi pathway. In addition, MOV10 has been found to co-localize with Ago1 and Ago2 in P bodies and stress granules. Interestingly, MOV10 also interacts with APOBEC3G and APOBEC3F in these cellular compartments.

Here, we report that MOV10 inhibits retrovirus replication. When it was over-expressed in viral producer cells, MOV10 was able to reduce the infectivity of HIV, SIV, and MuLV. Conversely, when MOV 10 expression was reduced by siRNAs, HIV-1 infectivity was increased. Consistently, silencing of MOV10 expression in human T cell line enhanced HIV-1 replication. Furthermore, we found that MOV10 interacts with HIV-1 NC and is packaged into virions. It blocks HIV-1 replication at a post-entry step. In addition, we also found that HIV-1 could suppress MOV10 protein expression to counteract this cellular resistance. All these results indicate that MOV10 has a broad antiretroviral activity that can target a wide range of retroviruses, and it could be actively involved in host defense to retroviral infection.

HIV-1 VIF BINDS TO APOBEC3G MRNA AND INHIBITS ITS TRANSLATION

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The HIV-1 viral infectivity factor (Vif) allows productive infection of non-permissive cells (including most natural HIV-1 targets) by counteracting the cellular restriction factor APOBEC3G (hA3G). The Vif-induced degradation of hA3G by the proteasome has been extensively studied, but little is known about the translational repression of hA3G by Vif, which has also been proposed to participate in Vif function.

To address the role of Vif in the translational regulation of hA3G, we characterized Vif binding to the full-length hA3G mRNA and to fragments corresponding to its coding or unstranslated regions (UTR). Filter binding assays and fluorescence titration curves revealed that Vif tightly binds to hA3G mRNA. Vif binding affinity was higher for the 3'-UTR than for the 5'-UTR, even though this region contained at least one high affinity Vif binding site. Several Vif binding sites were mapped in these regions by enzymatic footprinting. Finally, we analyzed the effect of Vif on hA3G translation in an in vitro transcription/translation assay and evidenced that Vif inhibits translation of hA3G mRNA when the 5'-UTR is present. Successive truncations in the 5' UTR revealed a structural motif containing at least one Vif binding site that was required for the regulation of hA3G translation by Vif, suggesting that the molecular mechanism of translational control is more complicated than a simple physical blockage of scanning ribosomes. Finally, analysis in transfected cells +/– proteasome inhibitors only slightly increased the hA3G levels, irrespective of the mRNA used to translate. Thus, our experiments strongly suggest that Vif also inhibits hA3G in transfected cells and that under our conditions this mechanism is more potent than the Vif-induced hA3G degradation.

HUMAN APOBEC4 – A NOVEL CELLULAR CO-FACTOR IN HIV REPLICATION

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Computational analysis pointed out a new member of the APOBEC-protein superfamily in 2005, APOBEC4 (1). APOBEC4 is present in mammals, birds and amphibia, while it could not be found in fishes so far. The tissue expression profile of the murine orthologue suggests that the protein might be highly expressed in mammalian testes (Novartis Gene Expression Atlas) and thereby be active in spermatogenesis. As numerous APOBEC3-proteins show antiviral activity against various retroviruses, human APOBEC4 was tested here for its potential antiviral activity against HIV-1. The reduction of infectivity reported for APOBEC3 proteins was not observed. In contrast, in a titration experiment using HIV-1wt, we found an APOBEC4-dependant increase of the virus titer. The same dependency was observed by measuring intracellular p24 concentration. The increase in nef ORF expression suggests that early viral products are affected in the same manner. A4 did not raise the LTR-driven expression and thus increases/stabilizes the HIV-protein production by a novel unknown way. Additionally, we were able to prove mRNA expression of endogenous A4 in 293T and in HeLa cells by PCR and sequencing. siRNA mediated down regulation of APOBEC4 resulted in reduced virus production. As editing activity of APOBEC proteins have already been described in detail, we also tested APOBEC4 for its potential editing properties. APOBEC-like editing of HIV or editing activity in the E. coli mutation assay was not detectable. Taken together, A4 seems to be recruited as a cellular co-factor for HIV replication.

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PRODUCTIVE REPLICATION OF VIF-CHIMERIC HIV-1 IN FELINE CELLS

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Non-primate animal models of HIV-1 infection are prevented by missing cellular cofactors and by antiviral actions of host defense factors. These blocks are profound in rodents but may be less abundant in certain Carnivora. Here we enabled productive, spreading replication and passage of HIV-1 in feline cells. We demonstrate that feline APOBEC3 (fA3) proteins are the principal non-receptor block to this virus. Feline fibroblasts, T cell lines and primary peripheral blood mononuclear cells supported early and late HIV-1 life cycle phases equivalently to human cells with the exception that produced virions had low infectivity. Stable expression of FIV Vif-GFP in HIV-1 entry receptor-complemented feline (CrFK) cells enabled robust spreading wild-type HIV-1 replication. FIV Vif interacted with all three fA3s, targeted them for degradation and prevented G to A hyper-mutation of the HIV-1 cDNA by fA3CH and fA3H. In contrast, HIV-1 Vif interacted with fA3s but did not cause their degradation and even paradoxically augmented restriction. SIVmac Vif prevented hypermutation by fA3CH and fA3H effectively but was less active than FIV Vif in fA3H restriction and degradation assays. HIV-1 clones that encode FIV Vif or SIVmac Vif (HIV-1^{VF} and HIV-1^{VS}) were then constructed. HIV-1^{VF} and HIV-1^{VS} replicated productively in HIV-1 receptor-complemented feline cells and could be passaged serially to uninfected cells. Thus, except for entry receptors, feline cells can supply all necessary dependency factors needed by HIV-1, and the main restriction can be countered by vif chimerism.

EVIDENCE FOR A PALEOVIRUS INFECTION IN CHIMPANZEE ANCESTORS

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The Apobec3 locus in humans encodes a family of cytidine deaminases (Apobec3A, B, C, DE, F, G and H) that inhibit the replication of retroelements during reverse transcription. The Apobec3 family members have a broad spectrum of strengths and targets, suggesting their adaptation to particular retroelements. Moreover, the adaptive evolution of these family members suggests that their sequence divergence between primates has been driven by genetic conflict with ancient pathogens. We previously characterized the rapid evolution of Apobec3G and 3H. However, the Apobec3 gene with the highest human-chimp divergence is Apobec3DE, a gene for which a clear role for in antiviral host defense has not yet been identified.

Using phylogenetic analysis by maximum likelihood, we found that much of the sequence divergence between human and chimp Apobec3DE is due to extensive positive selection in the chimpanzee lineage before divergence with bonobos, suggesting that the strength of the selective pressure was much greater in chimps than in humans. The amino acids that show the greatest evidence of positive selection cluster in the C-terminal domain of chimp Apobec3DE, near a putative packaging signal and Vif interaction domain, and near the N-terminus in human Apobec3DE, suggesting that the nature of the selective pressure on Apobec3DE was also very different between the two species. From this data, we hypothesize that a nucleocapsid-like factor or a Vif-like factor may have driven the adaptive evolution in chimp Apobec3DE.

While both human and chimp Apobec3DE are able to significantly restrict Alu and MusD, two distantly-related retrotransposons, the chimp ortholog is very active against lentiviruses, including HIV-1 and SIVagm (while human Apobec3DE has poor activity against both). These results further suggest that Apobec3DE has evolved to restrict a different pathogen, specifically an extracellular retroelement, in chimpanzees than in humans. We mapped the residues of Apobec3DE that are responsible for the differential human vs. chimp anti-viral activity to a region within the C-terminal domain. We are testing whether these amino acids alter packaging efficiency in human and chimp Apobec3DE. Additionally, we are comparing the sensitivity of human and chimp Apobec3DE to multiple lentiviral Vif proteins. These results point to a paleovirus infection in the ancient chimpanzee lineage approximately 2-5 million years ago that drove selection of chimp Apobec3DE, while having more minor effects on the human lineage.

CHARACTERIZATION OF THE BARRIER CONFERRED BY TYPE I INTERFERON ON THE EARLY STEPS OF HIV-1 INFECTION IN PRIMARY CELLS

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Type I interferon (IFN) induces blocks to infection by many viruses at various steps of their life cycles. It has been known for a long time that treatment of certain cell types with IFN confers a potent block to both the early and late steps of HIV-1 replication. However, the early block has remained poorly characterized and the identity of the anti-viral factor(s) that mediate this effect is unknown.

We have investigated in detail the effect of type I IFN on HIV-1 infection in cell types relevant to natural infection, including primary CD4+ T cells, monocyte-derived macrophages, as well as several monocytic and T cell lines. We have found that IFN treatment of macrophages, THP-1 cells, and to a lesser extent CD4+ T cells, has a profound effect on the outcome of HIV-1 infection, whereas the effect is much less pronounced in U937 cells and minimal in CEM, CEM-SS, HUT78, Jurkat or SupT1 T cell lines. Using a BlaM-Vpr entry assay, we have observed that viral entry is not affected by IFN treatment. More specifically, the block directly correlates with a strong decrease in the accumulation of viral cDNA. Interestingly, proteasome inhibitors rescue viral cDNA accumulation, reminiscent of what is seen with non-human isoforms of TRIM5α. We have also shown that IFN exerts potent anti-viral effects against a broad range of retroviruses, including diverse HIV-1 strains (IIIB, BK132, NL4-3, YU-2, Ba-L), HIV-1 derived lentiviral vectors (LVs), SIVmac and FIV LVs, as well as B-MLV.

Taken together, our results indicate that one or several effectors of the type I IFN response are able to block viral DNA accumulation of distant retroviruses. Interestingly, the restriction factors currently known to affect retroviral DNA accumulation, namely TRIM5α and APOBEC3G/F, are part of the IFN response. Considering the range of retroviruses that are restricted, we consider it unlikely that TRIM5α mediates this restriction, though we are currently investigating the potential role of different APOBEC3 family members. Interestingly, all the cells we have examined efficiently induce numerous interferon stimulated genes (ISGs) such as ISG15, OAS1 and PKR, indicating that a general loss of IFN-responsiveness does not underlie the lack of an IFN phenotype in T cell lines. Rather, we hypothesize that these cell lines are unable to induce expression of the critical anti-retroviral effector(s), contrary to macrophages, THP-1 or CD4+ T cells. Consequently, comparative transcriptomic analysis will be a tool of choice to help identify the ISG(s) responsible for the early block to retroviral infection.

IFITM PROTEINS INHIBIT HIV-1 INFECTION

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Interferon constitutes a major host defense mechanism against virus infection. Replication of human immunodeficiency virus type 1 (HIV-1) is substantially suppressed by interferonalpha in certain T cell lines such as SupT1. We have employed a shRNA-based knockdown approach to identify anti-HIV-1 ISGs (interferon stimulated genes) in interferonalpha-treated SupT1 cells. Among several ISG candidates thus identified is IFITM1 (interferon induced transmembrane protein 1). To assess the anti-HIV-1 activity of IFITM1 and its two homologues IFITM2 and IFITM3, we utilized the Tet-ON retroviral expression vector system to crease SupT1 cell lines that express these proteins in the presence of doxycycline. The results showed that expression of IFITM proteins profoundly inhibited HIV-1 replication in tissue culture. This inhibition was further shown to take place at the early steps of HIV-1 infection in the one-round infection experiment. Using the Vpr-BlaM-based HIV-1 virion fusion assay, we demonstrated that HIV-1 entry was impaired. Our data suggest that IFITM proteins act as a new group of cellular restriction factors that inhibit HIV-1 infection by targeting virus entry.

Jennifer Lu and Qinghua Pan contributed equally to this work.

K70Q ADDS HIGH-LEVEL TENOFOVIR RESISTANCE TO "Q151M COMPLEX" HIV REVERSE TRANSCRIPTASE THROUGH THE ENHANCED DISCRIMINATION MECHANISM

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HIV-1 carrying the "Q151M complex" (Q151Mc, A62V/V75I/F77L/ F116Y/Q151M in reverse transcriptase [RT]) shows multi-drug resistance (MDR) to FDA-approved nucleoside RT inhibitors (NRTIs) but has been considered susceptible to tenofovir disoproxil fumarate (TNV-DF).
We have isolated a Q151Mc-containing clinical isolate with phenotypic resistance to TNV-DF under TNV-DF-treatment. Genotypic and phenotypic analyses over the course of this patient's therapy suggested that resistance to TNV-DF emerges upon appearance of the previously unreported K70Q mutation in the Q151Mc background. Virological analysis revealed that the K70Q mutation by itself did not significantly affect resistance to TNV-DF and resulted in marginal resistance to didanosine (ddI) and lamivudine (3TC). However, addition of the K70Q mutation to the Q151Mc background enhanced resistance to all FDA-approved NRTIs, including TNV-DF (10-fold). Biochemical analysis showed that K70Q/Q151Mc RT does not become more efficient in ATP-based excision of chain-terminated primers. Pre-steady state kinetic analysis of the recombinant enzymes demonstrated that the K70Q mutation significantly affects incorporation of TNV-diphosphate (TNV-DP) into the nascent chain due to decreased TNV-DP binding affinity. Molecular modeling analysis suggested that the K70Q mutation may indirectly influence binding of the TNV-DP through interactions with residue K65.
Mutations at position 70 of RT have been known to confer NRTI resistance either a) by enhancing excision (through K70R), or b) by decreasing the incorporation rate of the inhibitor (kpol-dependent, through K70E). In this study, we present a third possible mechanism: c) by decreasing the binding affinity of the inhibitor (Kd-dependent, through K70Q). These findings highlight the remarkable ability of RT to use different mutations at this position to acquire NRTI resistance through three different mechanisms. The discovery of the novel mutational pattern (K70Q/Q151Mc) that causes high-level resistance to TNV as well as other NRTIs may have clinical implications in therapeutic strategies for NRTI-experienced patients with MDR mutations.

THE FEZ1 INTERACTING PROTEIN NEK1 IS A NEGATIVE REGULATOR OF HIV-1 INFECTION IN CULTURED CELLS

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Our previous work has shown that the neuronal specific factor FEZ1 (fasciculation and elongation protein zeta-1) blocks retroviral infection early in the viral life cycle, after reverse transcription but before nuclear entry. We also recently demonstrated that neurons unlike astrocytes and microglia naturally express high levels of FEZ1 and are correspondingly less susceptible to HIV-1 infection. FEZ1 overexpression was recently shown to be the cause of multi-lobulation of the nucleus, a phenotype frequently seen in leukemia cells. The same research group also demonstrated that FEZ1 binds and forms a functional complex with NIMA-related kinase 1 (NEK1) and CLIP-associated protein 2 (CLASP2) in a perinuclear region in mammalian cells. NEK1 and CLASP2 participate in cell cycle and cell division processes and localise to the centrosome. Here we examined FEZ1 overexpressing microglia CHME3 cells by fluorescence microscopy and confirmed that FEZ1 overexpression is the cause of multi-lobulation of the nucleus in these cells. Our preliminary data suggests that knockdown of endogenous NEK1 significantly increases the susceptibility of a neuronal cell line to pseudotyped human immunodeficiency virus type 1 (HIV-1) infection. Moreover, overexpression of NEK1 in HeLa cells showed an increased resistance of these cells to HIV-1 infection. As such, NEK1 and FEZ1 act as negative regulators of retroviral infection. We have generated truncation mutants of FEZ1 with the aim of identifying domains required for the inhibition of HIV-1 infection and binding to NEK1, which may actually be the same domain required for the formation of a larger FEZ1/NEK1 complex that affects nuclear architecture and HIV-1 entry.

XMRV IS NOT DETECTED IN QUEBEC PATIENTS WITH CHRONIC FATIGUE SYNDROME

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It was recently reported that 67% of US patients with chronic fatigue syndrome (CFS) show the presence of xenotropic murine leukaemia virus-related virus (XMRV) in peripheral blood mononuclear cells (PBMC). However, similar results could not be reproduced with CFS patients from other countries, the UK or the Netherlands.
We studied a Quebec cohort of sixty-two (62) CFS patients to determine whether XMRV is also associated with CSF in this part of the world. We used a PCR method to amplify gag- and env- specific XMRV sequences with PBMC DNA from CFS patients. None of these DNA samples tested gave a positive and/or reproducible signal. In addition, PHA + IL-2 activated PBMC from each patient were co-cultured with susceptible LNCaPs cells for 1.5-2 months. Virus particles were then assessed by an rA.dT assay. None of the 62 cultures gave a positive read-out with this assay. In addition, we could not detect viral footprints in activated PBMC by immunofluorescence with anti-p30 antibodies.
Therefore, it appears that XMRV footprints cannot be easily detected in Quebec CFS patients by the methods used. Possible explanations for these contrasting results could be: (a) the virus titers may be too low in PBMC to be easily detectable; (b) the virus may be divergent enough in our cohort to be undetectable with the approaches used; (c) or XMRV may not be associated with the development of XMRV in Quebec patients.

THE APOBEC3-VIF INTERACTION IS SEMI-PROMISCUOUS

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All lentiviruses except EIAV use the small accessory protein Vif to counteract the restriction activity of the relevant APOBEC3 (A3) proteins of their host species. Prior studies have indicated that the Vif-A3 interaction is species-specific. Here, using APOBEC3H (Z3)-type proteins from five distinct mammals, we report that this is generally not the case: some lentiviral Vif proteins are capable of triggering the degradation of both the A3 proteins of their normal host species and those of several of the other mammals. For instance, SIVmac Vif can mediate the degradation of the human and rhesus macaque A3H proteins and the cow A3Z3 protein, but not degradation of sheep, cat, or mouse A3Z3 type proteins. MVV Vif is also very promiscuous, degrading not only sheep A3Z3 but also the Z3-type proteins of humans, rhesus macaques, cows and cats. In contrast, the interactions between HIV Vif, BIV Vif, and FIV Vif appear specific to their host A3Z3 proteins. Taken together with the fact that all of these experiments were done with an HIV-based vector in human cells to minimize non-Vif/A3 experimental variables and that only 25 Vif/A3 combinations were surveyed, it is likely that all lentiviral Vif proteins will show significant degrees of cross-species A3 neutralization. Our studies also suggest that the Vif/A3Z3 interaction is relevant to lentivirus pathogenesis and that the flexibility of this interaction may facilitate zoonotic infections.

INSIGHTS INTO THE AFFECT OF HIV-1 TAT RNA SILENCING SUPPRESSOR ACTIVITY ON THE MIRNA PROFILE OF HUMAN LYMPHOCYTES

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The host RNA silencing machinery downregulates HIV-1 expression, presumably by specific miRNAs. Cell culture experiments have demonstrated host miRNAs that are detrimental to HIV-1, that Dicer downregulation increases HIV-1 virion production, and that HIV-1 Tat possesses RNA silencing suppressor (RSS) activity that counteracts translational repression of HIV-1. Comparison of Tat RSS with plant and animal virus RSS has demonstrated that Tat RSS activity is conserved across kingdoms, and Tat RSS is active at a step downstream of miRNA maturation. Herein trends in miRNA expression profiles observed in a 2008 study of 36 HIV-1 infected individuals were compared to miRNA expression profiles observed in CEMx174 lymphocytes infected with HIV-1NL4-3 or the K51A derivative strain deficient in Tat RSS activity. Unexpectedly, results identified similar profiles between the cell culture system and a cohort of 36 HIV-1 patients previously characterized, supporting the validity of a cell culture model to evaluate HIV-1 miRNA biology prior to targeted experiments in patient samples. Removal of Tat RNA silencing suppressor activity (K51A) affected the magnitude of a subset of miRNAs. Discussion will include the possible impact of Tat RSS activity on host miRNA activity, relation to steady-state miRNA level, and interplay of feedback on alternative target genes.

AN ANCESTOR COMES INTO VIEW: RECONSTRUCTION OF AN ACTIVE APOBEC3G GENE FROM ANCIENT PROCESSED PSEUDOGENES

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The Apobec3 (A3) loci vary significantly between different mammalian orders, showing both evidence of expansion and contraction in the number of paralogous genes as well as rearrangements, which reflect the selective histories of different genes. Old world monkeys share a common arrangement of seven genes within the locus in the order A3A through A3H. These are made up of monomeric or dimeric combinations of three paralogous sequences (Z1, Z2 and Z3) encoding zinc-coordinating proteins with deaminase activity. In contrast, the mouse has a single gene with a Z2-Z3 configuration. In addition to the seven genes in the A3 locus, all old and new world monkeys share a processed syntenic A3G pseudogene; in the human genome this sequence is located on chromosome 12 q23.3. The presence of this pseudogene in both old and new world monkeys indicates that the A3G locus was already present in the common ancestor around 35 million years ago. Attempts to detect the pseudogene by Southern blot analysis or PCR amplification in the DNA of the more distantly related lemur have so far been unsuccessful. Interestingly, the genomes of new world monkeys carry additional processed pseudogene copies of A3G. Some of these amplifications seem quite recent based on sequence similarity to the active A3G genes. There is no evidence of processed pseudogenes for any of the other monkey A3 genes based on the genomes of primates in the database.

We compared the common pseudogene of new and old world monkeys using sequences found in data bases as well as established by direct sequence analysis, and found that they all show overt inactivating mutations which interrupt the reading frame. We derived a consensus sequence with an open reading frame based on sequences from new and old world primates and generated an expression construct termed PsuA3G. The protein encoded by the initial construct was highly instable due to poly-ubiquitination and was consequently inactive in retroviral restriction. Using human/Psu A3G hybrids we identified the carboxy-terminal domain as the major determinant of the instability. A change of one residue (S267P) in the C-terminal domain of the PsuA3G protein resulted in its stabilization and reactivation. PsuA3G-S267P shows 2-fold less activity in restriction of HIVΔvif compared to human A3G. We are currently analyzing other point mutations in an attempt to further increase the activity of PsuA3G.

SPECIES DISTRIBUTION AND EVOLUTION OF TWO ENVELOPE PHENOTYPES OF CLASS II RETROVIRUSES

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We have previously shown that representative members of the Betaretrovirus genus— Jaagsiekte sheep retrovirus (JSRV) and mouse mammary tumor virus (MMTV)—share with lentiviruses the phenotype of non-covalently associated envelope (Env) subunits. Furthermore, subunit association can be predicted by the pattern of cysteine residues in the flexible region of the transmembrane (TM) domain, with a two-cysteine motif indicating non-covalent association and a three-cysteine subunit, covalent bonding between the TM and the surface subunit (SU) of Env. A look at the TM sequences of the human endogenous retroviruses (HERV), for instance, reveals that the HERV-K group contains the motif associated with non-covalent subunit association, and this prediction was supported by our demonstration that HERV-K108—a member of the HERV-K(HML-2) group, whose *env* reading frame is open and functional—indeed has non-covalently associated subunits. This finding indicates that the non-covalent phenotype first infected the ancestor of humans at least 55 million years ago, in the form of HERV-K(HML5).

We are currently analyzing the species distribution of elements with the non-covalent motif, and comparing phylogenies of these viruses with those of their hosts to gain insight into how this structure evolved in relation to its hosts over time. Preliminary results suggest that, while the non-covalent phenotype has been quite successful in infecting the germ-lines of mammals, the covalent phenotype has a broader species distribution. Information gained from this analysis should also be useful for fine-tuning the taxonomy of the Class II retroviruses in general and the Betaretrovirus genus in particular, which currently include viruses with both phenotypes.

A RANDOM SCREENING ASSAY FOR IDENTIFICATION OF INHIBITORS AND HOST CELL FACTORS INVOLVED IN HIV-1 MORPHOGENESIS

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Late stages in the replication cycle of HIV-1 are highly organized processes involving the interaction of viral components with complex cellular machineries which are currently only partly understood. Although recent high-throughput siRNA screens identified many putative host cell factors involved in HIV-1 replication, still only a limited number of essential cofactors for viral egress are known. To specifically address this question, we set up a simple assay suitable for random screening approaches, allowing both the identification of chemical compounds interfering with assembly or release of HIV-1 particles, and of important host cell factors by screening of siRNA libraries.
The protocol comprises transfection of 293T cells with a fluorescently labeled HIV-1 derivative, which carries an eYFP domain within the main viral structural protein Gag. This allows direct quantification of released particles by measurement of the YFP intensity in the cell culture supernatant. To control for potential differences in transfection efficiencies and cytotoxic effects, YFP intensities in the cell lysates were quantitated in parallel, and YFP intensity in the culture medium was normalized to the total fluorescence in the supernatant and cell lysate.
A primary screen was performed for a subgenomic siRNA library targeting 740 human kinases. After statistical analysis, candidates scoring positively were tested in a validation screen. Bioinformatics analyses of validated host factors are currently performed in order to identify potentially relevant protein pathways and networks. Thorough characterization of the functional role of such potential host factors may contribute to a better understanding of HIV-1 morphogenesis in the future.

MATURATION INDUCES A STRUCTURAL REORGANIZATION OF THE HIV-1 GAG POLYPROTEIN.

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The assembly of retroviruses is a highly ordered process that is driven by the multi-functional polyprotein Gag. In the immature stage Gag is responsible for packaging of cellular factors, self-targeting to the site of assembly, virus assembly and release of the virus particles from the infected cell. After the ordered assembly and release from the cell Gag is processed, which allows the individual domains of Gag to take on new roles as the structural domains that are responsible for creating a fully infectious particle. Even though a large amount of structural data is available on the mature domains of Gag, the mechanism that orchestrates the metamorphosis of Gag is still largely unknown.

Here we describe the conformation of the full-length HIV-1 Gag polyprotein and its cleavage products in solution and show that HIV-1 GAG changes conformation during maturation. We show that the immature full-length HIV-1 Gag polyprotein adopts a compact structure, which is mediated through the C-terminus of Gag. Comparison of the monomeric solution structure of the Gag precursor protein, processing intermediates and mature products revealed that a conformational epitope within the HIV matrix domain is not accessible in the precursor form but is exposed in the context of the processing intermediate and mature product. This same conformational epitope is not detected in immature HIV, but is readily recognized in mature infectious HIV.

To summarize, our results show that HIV Gag undergoes dramatic tertiary structure changes during maturation and we hypothesize that these conformational changes are likely to be important for the production of infectious HIV.

IDENTIFICATION OF HIV-1 VPU AS A LIPID RAFT PROTEIN AND ITS ROLE IN ENHANCED VIRION RELEASE

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Lipid rafts are ordered domains within the cell membrane, enriched in cholesterol and sphingolipids, and known to be critical for entry and egress of many viruses from the cell. HIV-1 is known to bud from lipid rafts and several HIV-1 proteins localize to these domains, including Env, Gag, and Nef. The HIV-1 Vpu protein has two major functions, downregulation of CD4 from the cell surface and enhanced virion release/relief of BST-2 restriction. As BST-2 is a GPI-anchored, lipid raft protein, we examined whether HIV-1 Vpu is a lipid raft protein. We found that approximately 10% of Vpu is consistently localized to detergent resistant membranes (DRMs) when exogenously expressed in the absence of other viral proteins, with or without a C-terminal EGFP tag, and also from virus-infected cells. A Vpu protein from a clinical isolate of HIV-1 subtype C was isolated from resistant fractions in similar ratios. Cholesterol depletion of cells with lovastatin and methyl-β-cyclodextrin removed Vpu from DRMs indicating the specificity of Vpu partitioning into DRMs. A Vpu protein with a scrambled transmembrane domain previously described was no longer found in detergent resistant fractions. A series of alanine mutations in the transmembrane and proximal cytoplasmic domain were analyzed to determine if a specific portion of these domains was involved in raft targeting. The ability of mutant Vpus to partition into DRMs was correlated with the ability of Vpu to enhance virion release from HeLa cells in a single round replication assay. This work supported by NIHAI51981.

VPR (HIV-1) FUSED TO APOBEC3C ALTERS ITS RESTRICTION PROPERTIES BUT NOT THE SUB-VIRAL LOCALIZATION

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The APOBEC3 family of cytidin deaminases protects vertebrates from viral pathogens. Although the APOBEC3 protein family is very homologues sharing a characteristic motif for zinc-coordination, the specificity of each APOBEC3 differs from the others. Several APOBEC3 proteins (e.g. A3F and A3G) restrict Human immunodeficiency virus (HIV) replication in the absence of the viral infectivity factor (Vif) protein. Another member, APOBEC3C(A3C) so far is only known to be active against Simian immunodeficiency virus (SIV) and LINE-1 retrotransposons, but not against HIV.
Our study confirmed that A3C has no antiviral effect against HIV-1 Δvif although it is incorporated into viral particles. Aguiar et al. (JBC 2008) showed that fusion of VPR (HIV-1) to the N-Terminus of A3A resulted in antiviral activity against HIV-1 due to a change in sub-viral localization of the fusion-protein. We tested this idea also for A3C, and observed a strong antiviral activity against Δvif HIV-1. Interestingly, also wt HIV-1 was inhibited by VPR-A3C indicating a block of A3C interaction with Vif. This dose-dependent effect was shown for VPR fused to either the N- or the C-Terminus of A3C. We speculated that the interaction of VPR with the nucleocapsid (NC) protein of HIV-1 could alter sub-viral localization and target A3C directly to the viral core or RNP, respectively. But, unexpectedly, we could not see any difference in sub-viral localization of the fusion-protein compared to wtA3C and wtA3G. In pull-down assays of NC (HIV-1) with A3C and A3G we observed equal binding properties for both proteins. In ongoing experiments, we characterized the relevance of small A3C-interacting RNAs like 5.8S or 7SK RNA for the different activities of the A3 constructs. Our findings implicate, that enhancing the antiviral activity of the broadly expressed A3C would likely repress HIV-1 replication in patients and thus should be considered as a novel approach for treatment.

LIPID RAFT, TETRASPANIN-ENRICHED MICRODOMAIN, AND GAG DISTRIBUTION DURING HIV-1 ASSEMBLY

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The nature of biological membranes is fundamental to the study of assembly and egress of enveloped viruses. The current consensus is that the plasma membrane (PM) is heterogeneous, consisting of diverse microdomains. This partitioning of membrane components is regulated by lipid-lipid, protein-protein, and protein-lipid interactions to compartmentalize cellular processes. Previously, we and others have reported that HIV-1 Gag co-fractionates with detergent resistant membranes, and is sensitive to cholesterol depletion. More recently, two types of microdomains, lipid rafts and tetraspanin-enriched microdomains (TEMs), have been proposed to be platforms of HIV-1 assembly.

Lipid rafts are dynamic, sub-microscopic domains enriched in sterols, sphingolipids, GPI-anchored proteins. TEMs are membrane microdomains organized by oligomerization of tetraspanin proteins, such as CD81, CD9, and CD63. Importantly, despite some similarities, lipid rafts and TEMs can be distinguished by a variety of assays, and are therefore thought to be distinct classes of microdomains. Yet, it has been reported that Gag associates with both lipid rafts and TEMs, suggesting that Gag may induce the coalescence of these otherwise distinct microdomains during HIV-1 assembly.

In this study, using an antibody-mediated co-patching assay, we observed the cell-surface distribution of canonical lipid raft markers, such as GPI-anchored proteins and influenza HA, and tetraspanin proteins, in the presence or absence of Gag derivatives. We confirm previous observations that lipid raft and TEM markers segregate upon patching, and therefore appear to constitute distinct microdomains in the absence of Gag. However, we demonstrate that Gag does induce the coalescence of lipid rafts and TEMs. Using a panel of Gag mutants designed to alter Gag acylation, $PI(4,5)P_2$ affinity, multimerization, and ability to induce membrane curvature, we investigate the molecular determinants of this coalescence. Preliminary data indicate that Gag membrane binding is necessary, but a variety of alternate membrane binding domains are sufficient to allow Gag-mediated microdomain coalescence.

The ability of Gag to reorganize membrane microdomains may have important implications for viral fitness. If Gag-mediated reorganization of microdomains increases the affinity of Gag for these microdomains, this could confer cooperativity on Gag membrane and microdomain association, possibly favoring multimerization and assembly. Additionally, Gag-induced microdomains may facilitate the incorporation of important viral or cellular factors, such as viral glycoproteins or restriction factors (e.g. tetherin).

MONITORING CELLULAR METABOLIC ALTERATIONS DURING HIV-1 PRODUCTION

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U937 is a promonocytic cell line that can be differentiated into monocyte/macrophages with PMA treatment. U1/HIV-1, a subline of U937, has integrated HIV-1 proviral DNAs and initiates HIV-1 production upon PMA treatment. Here we employed U937 and U1/HIV-1 models to investigate the cellular metabolic alterations during viral production. First, we found that 100 nM PMA treatment for 24h worked well leading to better adherence of the U937 cells as compared to U1/HIV-1 cells. Next we examined p24 level at the cellular level by FACS analysis and found over 50% of the U1/HIV-1 cells expressed p24 by 48h. We found an increase p24 level by ELISA in the supernatants between 24h and 72h after PMA treatment. Cell death was examined by Trypan blue exclusion and was found to be very low at 24h, but steadily increased to 96h, the end point of these studies. U937 appears more susceptible to death after PMA as compared to U1/HIV-1 cells. With this finding, we examined the steady state profiles of various cellular metabolic intermediates using LC/tandem-MS at 24h and 48h post PMA treatment, in order to monitor metabolic alterations during viral production. Moreover, we will monitor HIV-1 induced metabolic changes by using a flux measurement with C13-labeled glucose. This system will be used to examine alterations in metabolism for glucose consumption and energy generation induced by HIV-1 production.

DEFINING THE RELATIONSHIP BETWEEN HIV-1 REVERSE TRANSCRIPTION AND UNCOATING

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HIV-1 replication begins with fusion of the viral particle with the target cell membrane, resulting in the release of the viral core into the cell. Once in the cytoplasm, the p24 capsid protein ($p24^{CA}$) disassembles from the core by a process called uncoating. Uncoating is required for viral replication, yet little is known about the kinetics and factors involved with the process. Previous studies have shown that mutations in $p24^{CA}$ can affect reverse transcription. Reverse transcripts have been detected in viral particles that contain $p24^{CA}$ using microscopy. Collectively, these data suggest that the processes of uncoating and reverse transcription may be functionally linked. Here, we have examined the timing and role of reverse transcription in HIV-1 uncoating.

We developed an uncoating assay which uses fluorescent microscopy to examine the association of $p24^{CA}$ with viral cores in the cytoplasm of infected cells. The membrane label S15-mCherry was incorporated into HIV-1 virions containing GFP-Vpr. HeLa cells were spinoculated with dual labeled virions at 17°C, temperature shifted to 37°C to allow infection, and then fixed and stained with an antibody to $p24^{CA}$. A subsequent loss of mCherry signal correlates with productive viral fusion, and the percentage of fused virions that retain $p24^{CA}$ was determined. We found that $p24^{CA}$ remained associated with the viral core after fusion and was gradually lost over time. This assay was validated using a set of $p24^{CA}$ mutants that previously have been shown to alter capsid stability and reduce infectivity. A correlation between the results of the uncoating assay and previous biochemical uncoating data was observed. Interestingly, four hours post-infection wildtype and $p24^{CA}$ mutants uncoated to a similar extent. These results indicate that an uncoated viral core is not solely sufficient for viral replication and that alterations in the rate of uncoating soon after fusion can impact viral replication. Finally, the effect of reverse transcription on uncoating was examined by treating cells with the reverse transcriptase inhibitors AZT and nevirapine. In treated cells we observed an increase in the percentage of virions associated with $p24^{CA}$ over time compared to no treatment. We complemented these experiments using TRIM abrogation and washout assays. These assays are based on the ability of the restriction factor TRIMCyp to inhibit HIV-1 replication through binding to an intact viral capsid. Treatment with nevirapine enhanced the ability of HIV-1 to saturate TRIMCyp restriction. These data correlate with observations from the uncoating assay in which uncoating was prolonged when reverse transcription was inhibited. Therefore, these results further support the idea that the processes of uncoating and reverse transcription are linked and suggest that steps in reverse transcription may be required for uncoating.

CHARACTERISTICS OF PORCINE ENDOGENOUS RETROVIRUS (PERV) WITH DEFECTIVE GENES PRODUCED FROM SNU MINIATURE PIGS

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Xenotransplantation using pig organs and tissues is considered as the possible answer to overcome the increasing needs for human transplantation donors. However porcine endogenous retrovirus (PERV) received much attention due to its unique nature of being endogenously present in every porcine tissue and due to its ability to integrate into human genome. There has been no evidence that PERV infection is associated with human diseases. It is necessary to analyze the construct and sequences of PERV genomes for the assessment of the capability of the self replication in cells after transmission.

It was calculated from the standard curve with real time PCR that 860 copies of *gag* and 55 copies of *pol* were present in a peripheral blood mononuclear cell (PBMC) of SNU miniature pig, which means that many defective PERVs are present in pig cells in genomic level. PCR amplification of PERV genes spanning of mid-5' LTR and 3' end of *env* in the chromosomal DNA of PBMC of SNU miniature pig revealed products with the approximate molecular weight of 7.5, 4.0 and 3.5 kDa. Gene products were cloned into Topo XL cloning vector, and their sequences were analyzed. The cloned PERV genes were classified into 6 groups according to the deleted or inserted genome patterns and the sizes of constructs. Group I was comprised of clones with full-length genes, group II to V clones with the variably and partially defective genes in *pol*, *env*, or *gag-pol* junction, and group VI clone with the inserted chromosomal sequences in *gag* portion. The sequence alignment suggested that most of the defective region in clones of group II to V was formed by the mechanism of splicing. The sequence analysis revealed that the cloned PERV genes in all the groups contained the presumed packaging signal sequences, from mid-5' LTR to 5' portion of *gag*, as compared with those from other retroviruses. It was detected mainly defective sizes of PERV genes when RT-PCR was performed in the cellular mRNA and virion RNA from PHA- and PMA-stimulated PBMC of SNU miniature pig with the primer set for the full-length PERV genes. It seemed a long time ago that PERV virion assembled with the defective genes could be produced and transmitted to germ line cells where the defective PERV genes were integrated into chromosomal DNA and handed over to offspring forever.

In conclusion, it would be very difficult for PERV from SNU miniature pig to replicate by itself in a cell after transmission if it is not complemented with other retroviral components in the PERV-transmitted cell.

Intrinsic Restriction Activity by APOBEC1 against the Mobility of Autonomous Retrotransposons

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The ability of mammalian cytidine deaminases encoded by the *APOBEC3 (A3)* genes to restrict a broad number of endogenous retroelements and exogenous retroviruses, including Murine Leukemia Virus (MLV) and human immunodeficiency virus (HIV)-1, is now well established. The RNA editing family member apolipoprotein B (apo B)-editing catalytic subunit 1 (APOBEC1; A1) from a variety of mammalian species, a protein involved in lipid transport and which mediates C-to-U deamination of mRNA for apo B, has also been shown to modify a range of exogenous retroviruses, but it's activity against endogenous retroelements remains unclear. Here we show in cell culture based retrotransposition assays that A1 family proteins can also reduce the mobility and infectivity potential of LINE-1 (long interspersed nucleotide sequence-1, L1) and long-terminal-repeats (LTR) retrotransposons (or endogenous retroviruses) such as murine intracisternal A-particle (IAP) and MusD sequences. The anti-L1 activity of A1 was mainly mediated by a deamination-independent mechanism, and was not affected by subcellular localization of the proteins. In contrast, the inhibition of LTR-retrotransposons appeared to require the deaminase activity of A1 proteins. Thus, the AID/APOBEC family proteins including A1s employ multiple mechanisms to regulate the mobility of autonomous retrotransposons in a wide range of mammalian species.

WILD-DERIVED MOUSE STRAIN (MUS PAHARI) AS A SMALL ANIMAL MODEL FOR XMRV INFECTION

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Xenotropic murine leukemia virus (MLV)-related virus (XMRV) is a novel human retrovirus recently identified in patients with prostate cancer. Although XMRV infects human cells by targeting XPR1 cell surface receptor, it cannot infect cells from laboratory mice. Recently, Dr. Kozac's group has demonstrated that cells from certain wild mouse strains are permissive to xenotropic mouse gammaretroviruses. Here, we examined whether a wild mouse strain, Mus pahari, can serve as a small animal model for XMRV infection. First, we examined the susceptibility of primary Mus pahari fibroblast cells to XMRV. When the cells were infected with XMRV, they became XMRV Gag-positive and produced 2 x 10^5 IU/ml of cell-free virions. Mus pahari cell-derived virus showed no notable mutation. These observations indicate that Mus pahari-derived cells support early and late phases of XMRV replication. Next, we examined susceptibility of Mus pahari to XMRV in vivo. Single injection of XMRV induced high levels of XMRV neutralizing antibodies (>320) in 9 out of 10 injected mice as early as 2 weeks after injection. XMRV Env-specific antibodies were detected in the plasma samples obtained at 2 weeks post injection, while strong Env- and Gag-specific antibodies were detected at 2 to 3 months after injection. When the biodistribution of XMRV was examined, spleen (5 out of 9), blood (4 out of 9) and brain (4 out of 9) were found to have high copies of viral DNA. Sequencing analysis confirmed the amplified fragments from blood and spleen as XMRV. By co-culturing mouse blood cells with 293T cells, we were able to isolate XMRV from four XMRV-infected mice. These data suggest the lymphotropic nature of XMRV in this mouse strain. Unexpectedly, DNA extracted from a brain sample of an uninfected mouse was shown to be positive for XMRV by the realtime PCR assay. Subsequent studies, however, identified another MLV, which is closely related to a previously reported wild mouse isolate CasE#1, suggesting circulation of an exogenous CasE#1-like MLV strain in Mus pahari. In summary, our data demonstrate the possible use of this wild mouse strain to study XMRV infection in vivo, although we may need to pre-screen animals for CasE#1-related virus infection.

CELL-CELL TRANSMISSION ALLOWS HTLV1 TO CIRCUMVENT TETHERIN RESTRICTION

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The recently discovered tetherin enlarges a group of cellular proteins with antiviral activity. Acting non-specifically, tetherin is capable to prevent release of at least four virus families. In response to this challenge, viruses developed different approaches to overcome tetherin activity: Vpu in HIV, K protein of KSHV, HIV2 and SIV envelopes, envelope glycoprotein in Ebola virus, and SIV Nef.
It is understood that tetherin keeps HTLV1 attached to the cell surface, but it is unknown by what mechanism HTLV1 escapes tetherin retention. To answer the question of possible anti-tetherin activity of HTLV1 we compared the level of tetherin expression in non-infected and HTLV1-chronically infected T-cell lines. The level of tetherin expression in HTLV1-chronically infected cell lines was higher than in non-infected, suggesting that HTLV1 does not have a mechanism to downregulate tetherin. The finding that GPI-truncated tetherin is incorporated into VLPs indicates that HTLV1 does not remove tetherin from the sites of viral release. While tetherin expression severely affects infectivity in cell-free assays, its effect on cell-cell transmission (more typical for HTLV1) is much less profound. Fluorescent staining reveals colocalization of tetherin and HTLV1 Gag in cell-cell contact areas established between Jurkat and HTLV1-chronically infected MS9 cells. We did not observe polarization of tetherin in Jurkat cells expressing only HTLV1 Gag or only Tax. Tax causes polarization of a cell and directs Gag toward the cell-cell contact area. The accumulation of tetherin in the cell-cell contact area is a result of the polarized release of viral particles. It is probable that tetherin, in tight cell-cell contact area, does not prevent transmission of HTLV1 to the target cell.

$PI(4,5)P_2$ IS NOT ESSENTIAL FOR PROPER HTLV-1 GAG LOCALIZATION AND EFFICIENT VLP RELEASE IN HELA CELLS

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The Gag polyprotein is necessary and sufficient for retroviral assembly. HIV-1 assembles predominantly on the plasma membrane (PM). The basic cluster of amino acids in matrix (MA) domain of HIV-1 Gag interacts with phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$], a phospholipid that is enriched on the PM. This interaction is essential for PM-specific HIV-1 assembly in HeLa cells. As many retroviral Gag also use basic amino acids in MA for membrane binding, $PI(4,5)P_2$ or other acidic lipids may be the common cellular factors involved in Gag targeting among retroviruses. Unlike HIV-1 Gag, Human T-lymphotropic virus type-1 (HTLV-1) Gag localizes to the intracellular compartments in HeLa cells. To study the role of HTLV-1 MA in Gag localization and virus assembly, we constructed a Gag chimera, by replacing the MA of HIV-1 Gag with that of HTLV-1 (HTMA). This Gag derivative, along with HIV-1 and HTLV-1 Gag, were expressed by CMV-promoter-driven plasmids. We compared the localization and VLP release efficiency of each Gag construct in HeLa cells. HIV-1 Gag localized mostly to the PM, but in the presence of 5ptaseIV, which depletes cellular $PI(4,5)P_2$, it displayed hazy cytosolic signal. Consistent with the lack of PM localization, VLP release was reduced 5 fold when compared to cells expressing inactive 5ptaseIV, 5ptaseIVΔ1, that lacks the catalytic domain. In contrast, both HTLV-1 and HTMA Gag localized to intracellular compartments in the presence or absence of 5ptaseIV, and the VLP release was only modestly reduced upon 5ptaseIV expression. To examine the contribution of $PI(4,5)P_2$ in membrane binding of HTLV-1 and HTMA Gag, we carried out an in vitro liposome binding assay. Consistent with previous data, HIV-1 Gag bound to liposomes in the absence of $PI(4,5)P_2$ poorly, whereas it bound 9-fold more efficiently to $PI(4,5)P_2$-containing liposomes. On the other hand, both HTLV-1 and HTMA Gag were able to bind efficiently to non-$PI(4,5)P_2$-containing liposomes, and the binding efficiency increased only 2 fold when $PI(4,5)P_2$ was present in the liposomes.
Altogether, these results indicate that MA of HTLV-1 determines the site of assembly. In addition, our data suggest that $PI(4,5)P_2$ plays a less important role in the assembly of HTLV-1 and HTMA compared to that of HIV-1. We seek to identify the key factors that determine the localization of HTLV-1 Gag.

STRUCTURE-GUIDED MUTAGENESIS OF APOBEC3G REVEALS FOUR LYSINE RESIDUES CRITICAL FOR HIV-1 VIF-MEDIATED UBIQUITINATION/DEGRADATION

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APOBEC3G (A3G) is a host cytidine deaminase that serves as a cellular defense protein and a potent inhibitor of HIV-1 replication. Abrogation of A3G function involves the HIV-1 Vif protein, a specific substrate receptor for ubiquitination that binds A3G. More specifically, Vif facilitates ubiquitination of A3G by forming an E3 complex consisting of Cullin5-ElonginB/C-Rbx2-E2, which targets A3G for rapid proteasomal degradation. Yet, despite our knowledge that these proteins interact with each other in the complex, the details of how the complex dictates A3G ubiquitination and subsequent degradation have remained unclear.

Here, extensive mutagenesis, guided by a structural model of full-length human A3G, has allowed us for the first time to identify four Lys residues (Lys297, 301, 303, and 334) that are required for HIV-1 Vif-dependent ubiquitination and degradation. Remarkably, substitution of Arg for Lys at these residues (the mutant is termed super A3G [S-A3G]) confers Vif resistance and restores A3G's antiviral activity even in the presence of Vif. In addition, we found that the interaction between WT A3G and S-A3G with Vif is very similar and that S-A3G is incorporated into WT and vif-deficient viral particles to the same extent. Interestingly, the Vif sensitivity of S-A3G was changed by addition of the C-terminal tags, indicating that Lys residues in the C-terminal tag can serve as ubiquitination sites. In our A3G structure model, the critical four residues cluster at the C-terminus, which is opposite to the known N-terminal A3G-Vif interaction region containing the $_{128}$DPD$_{130}$ motif. Moreover, HIV-2 and SIVmac Vif proteins target the same Lys residues in A3G as HIV-1 Vif. Collectively, these findings suggest that spatial constraints imposed by the E3 complex and especially the rigid scaffold Cullin5 protein may be an important determinant for Vif-dependent A3G ubiquitination/degradation.

THE POCKET IN THE N-TERIMINAL REGION OF APOBEC3G IS IMPORTANT FOR INTERACTION BETWEEN APOBEC3G, RNA, AND HIV-1 VIF.

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Background: Host restriction factor A3G blocks HIV-1 replication by inducing G to A hypermutation. HIV-1 Vif counteracts A3G by promoting its degradation via the ubiquitin-proteasome pathway. Previous studies have revealed that the Arg24 residue is important for its dimerization and virion incorporation which is mediated through interaction with viral or non-viral RNAs. Recently, we have also demonstrated that PKA binds and specifically phosphorylates A3G at Thr32 which interacts with Arg24 and that its phophorylation would stabilize this interaction, blocking its binding to Vif. We tested the possibility that the Arg24 residue of A3G is involved in the binding to Vif.
Method: We examined the protein binding between A3G and Vif with co-immunoprecipitation assays. We used VSV-G pseudotyped luciferase leporter viruses to assess the anti-HIV-1 activity of A3G and its mutants.
Results: We made several mutants at the position 24 of A3G, in which a residue was replaced by negatively charged or neutral amino acids and analyzed the binding of these mutants to Vif. R24A-A3G was still able to bind Vif, whereas R24E-A3G not, suggesting that the charged residue at the position 24 is important for interaction between A3G and Vif. In additon, the R24A mutant associated with hY or 7SLRNA less efficiently than the wild type and this was further impaired by the R24E substitution. The R24A and R24E mutants oligomerized less efficiently than the wild type. We conducted computer-assisted structural modeling of the A3G N-terminal domain including the wild-type, R24A, and R24E. Thermodynamically optimized three dimensional structures showed that the N-terminal region of A3G formed the pocket surrounded by Y22, R24, and R122 residues.
Conclusions: These data suggested that the pocket in the N-terminal region of A3G is constructed with Y22, R24, and R122 residues and the interaction between these residues is important for association of RNA, leading to oligmeriation and Vif binding.

MUTATIONS IN THE HINGE REGION OF HIV-1 CAPSID THAT YIELD NON-INFECTIOUS VIRIONS WITH CONICAL CORES

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The hinge or linker region between the N- and C-terminal domains of HIV-1 CA consists of residues S146, P147, T148, S149, and I150 and is flanked by residues Y145 and L151. To investigate the role of these residues in maintaining CA structure and function, we mutated each residue to Ala, with the exception of P147, which was changed to Leu. The mutants all produced particles, but only S146A and T148A had substantial infectivity. Thin-section EM revealed that three of the five non-infectious mutants, Y145A, I150A, and L151A had eccentric or centric cores and no conical cores. Surprisingly, two other mutants in this group, P147L and S149A, had a mixed population of conical cores (~50% of the WT value) as well as aberrant cores. The distinctive nature of these two mutants was also apparent in other assays. When viral DNA products were assayed in infected cells by qPCR, both mutants synthesized about 10-fold less DNA than WT. In strong contrast, I150A made ~10^4-fold less DNA than WT. We isolated virus cores and determined the amount of RT and CA retained in core fractions of sucrose gradients. RT values were on average ~35% of the WT level for P147L and S149A, whereas for the other non-infectious mutants the values were no more than 12% of WT. Retention of CA was more limited: for P147L and S149A, ~20% of the WT value was measured; and baseline levels were observed for Y145A, I150A, and L151A. Taken together, our results clearly demonstrate that perturbation of the CA protein in the hinge region leads to core instability and distortion of core architecture, with downstream effects on reverse transcription and infectivity. Attenuation of these effects in P147L and S149A is intriguing and is currently under investigation. This work was funded in part with federal funds from NCI, NIH, under contract HHSN261200800001E.

MOLONEY MURINE LEUKEMIA VIRUS GAG AND GAG-POL POLYPROTEINS ARE EQUALLY CAPABLE OF MEDIATING GRNA PACKAGING

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The nucleocapsid (NC) domain of the Moloney murine leukemia virus (MLV) Gag polyprotein is the determinant of genomic RNA (gRNA) packaging specificity. Of the ~2000 copies of Gag in an MLV virion, only a about a dozen appear required for specific gRNA recruitment (1). This suggests that the ~2000 NC domains are partitioned into at least two functional classes: those that engage in high-affinity interactions with gRNA, and those that do not. Testing phenotypically mixed virions generated by co-expressing WT and W35G NC Zn finger mutant MLVs confirmed that virions can package WT levels of gRNA when amounts of packaging-competent NC are reduced 4 to 10 fold, with packaging cut roughly in half for virions with only 5% WT NC. We next explored whether the Gag-Pol polyprotein, which is produced via termination suppression at a rate of about one Gag-Pol per twenty Gags, can function as well as Gag as the source of packaging-competent NC. This question was answered using two separate plasmids, pGag^{only} and pGag-Pol^{only}, that each expressed only a single polyprotein. It has previously been established that MLV virus-like particles can form when Gag is expressed alone, but not when only Gag-Pol is expressed. When cells were co-transfected with 20:1 ratios of WT pGag^{only} and WT pGag-Pol^{only}, complementation was observed, and served as a baseline for subsequent studies. Co-transfecting 20:1 ratios of WT pGag^{only} and W35G pGag-Pol^{only} yielded virions as infectious as those where both pGag^{only} and pGag-Pol^{only} were WT. 20:1 ratios of W35G pGag^{only} and WT pGag-Pol^{only} also complemented, albeit to a significantly reduced level. To more accurately assess the effects of NC polyprotein context on packaging, phenotypically mixed virions generated by transfecting 19:1:1 ratios of W35G pGag^{only}, WT pGag^{only} and W35G pGag-Pol^{only}, respectively, were compared to virions produced by 20:1 ratios of W35G pGag^{only} and WT pGag-Pol^{only}. Under these conditions, where the amount of WT NC was the same but its polyprotein context differed, titers were indistinguishable. Taken together, our results suggest that Gag and Gag-Pol can serve equally well to package MLV gRNA.

(1) Miyazaki et al. (2010) *J Mol Biol* 396:141-152.

TRIPARTITE-MOTIF (TRIM) 22 INTERFERES WITH NFAT-1-DRIVEN GENE EXPRESSION: IMPLICATIONS FOR HIV-1 TRANSCRIPTION.

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We have recently observed that endogenous TRIM22 is responsible for impairing HIV-1 long terminal repeat (LTR)-driven gene expression in a subset of promonocytic U937 cells non permissive for viral replication (A. Kajaste-Rudnitski et al., submitted). HIV transcription is regulated by a variety of cis-acting DNA sequence elements within the proviral LTR, responsive to specific host transcription factors. With the aim of identifying which of these factors could be involved in the TRIM22-mediated inhibition of HIV-1 transcription, an HIV-1 LTR Luciferase (Luc) reporter construct was transfected in 293T cells along with increasing amounts of TRIM22-expressing plasmid, followed by stimulation with Tumor Necrosis Factor-α (TNF-α), phorbol myristate acetate (PMA), either alone or in combination with ionomycin (I). Increasing amounts of TRIM22 expressing plasmid did not affect the HIV LTR basal transcription, however, PMA+I-induced HIV-1 LTR activation was inhibited down to basal level transcription. Since NF-κB, NFAT-1 and AP1 are among the most potent activators of HIV-1 LTR driven transcription, individual Luc reporter constructs were transfected in 293T cells along with increasing amounts of TRIM22-expressing plasmid, followed by stimulation with TNF-α, PMA, I or PMA+I combination. TRIM22 did not affect either NF-κB or AP-1-driven basal or stimulated Luc expression. In contrast, PMA+I stimulation of NFAT-1-driven Luc expression was inhibited by TRIM22 down to basal level transcription (from 6791±923 RLU upon PMA+I stimulation to 1890±207 RLU for PMA+I stimulation in presence of TRIM22 vs. 1832±149 RLU for basal activity). Stimulation with PMA alone did not induce NFAT-1-driven Luc expression, while Ionomycin did, although to a lesser extent than what observed in the presence of PMA+I. Interestingly, TRIM22 was able to inhibit also this activation. Overall, our results strongly indicate that TRIM22-mediated inhibition of HIV-1 transcription occurs through impairment of NFAT1-dependent activation of the proviral LTR. We are currently investigating whether a direct interaction between TRIM22 and NFAT-1 occurs and whether other molecular partners are involved.

POTENT INHIBITION OF HIV-1 LATE EVENTS BY INI1 MUTANTS DEFECTIVE FOR BINDING TO SIN3A ASSOCIATED PROTEIN (SAP18)

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INI1/hSNF5 is a host protein that directly binds to HIV-1 integrase (IN) and is selectively incorporated into HIV-1 virions. The minimal IN-binding fragment of INI1, S6, potently inhibits HIV-1 late events (Yung et al. Nat. Med. 2003). INI1 and IN directly bind to SAP18 (Sin3a associated protein 18kD), a component of the HDAC1 complex, and selectively recruits a Sin3a-HDAC1 complex into HIV-1 (but not SIV) virions in an HIV-1 IN-dependent manner (Sorin/Cano et al PLoS pathogens 2009).

To determine the significance of the INI1-SAP18 association, we isolated a panel of SAP18 interaction defective mutants of INI1 (SID-INI1*) using a reverse yeast two-hybrid system. Two mutants [INI1(G66A,V333E) and INI1(N149S,R239I)] that were stably expressed in both yeast and mammalian cells and selectively defective for binding to SAP18, but not for interaction with IN or c-MYC, were used for further study. Similar to our earlier report of transdominant negative effect of S6, co-transfection of SID-INI1* mutants along with HIV-1 viral vectors resulted in potent inhibition of HIV-1 particle production, 100-1000 fold, compared to either the wild type or the empty vector control. Expression of SID-INI1* resulted in 4-6 fold reduction in intra-cellular viral mRNA but ~100-fold reduction in viral protein levels. Determining the ratio of intracellular p24 to virion associated p24 indicated a further block in particle release. Analysis of LTR transcription indicated that while INI1 repressed basal as well as Tat-mediated LTR transcription, SID-INI1* mutants did not repress transcription, consistent with the recruitment of HDAC1 complex (and thus repression) by wild type INI1 but not by the SID-INI1*. Lastly, expression of SID-INI1* mutants did not affect nuclear export of viral RNA. These results together indicate that SID-INI1* mutants do not block viral transcription or RNA transport but block multiple steps of late events including viral RNA stability and viral protein translation and/or stability, assembly and particle release. These studies, for the first time, indicate that INI1-SAP18 interaction is required for late events of HIV-1 replication and that mimicking the effect of SID-INI1* mutants can be a new strategy to potently inhibit HIV-1 replication.

TRANSFORMATION TROPISM IS CONFERRED BY BOTH THE SURFACE AND TRANSMEMBRANE COMPONENTS OF HUMAN T LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) ENVELOPE

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Human T lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) are capable of transforming and immortalizing T lymphocytes. HTLV-1 is highly pathogenic and causes ATL and HAM/TSP. HTLV-2 is rarely pathogenic and sporadically associated with neuropathies. Both in vitro and in vivo studies indicate that HTLV-1 has a preferential tropism for CD4+ T cells while HTLV-2 for CD8+ T cells, yet the precise mechanisms for this differential tropism is poorly understood. We have already shown that the transformation tropism maps to the viral envelope. The viral envelope has two glycoproteins – surface (SU), which binds to cell receptor and transmembrane (TM), which triggers the fusion of the viral and cellular membranes, facilitating virus entry. Therefore, we aimed to further map the molecular determinant of the viral envelope, SU and TM components, of HTLV-1, in the context of the entire provirus for their contribution to the transformation tropism. HTLV-1 proviral recombinants with the HTLV-2 env sequences (SU2 or TM2) were generated. Both HTLV-1/SU2 and HTLV-1/TM2 recombinants showed Tax activity in transiently transfected cells, and produced p19 Gag levels that were proportional to wt virus in both transiently and stably transfected cells. Both HTLV-1/SU2 and HTLV-1/TM2 recombinant viruses were replication competent and transformed T cells in vitro. T cell phenotyping of these transformed T cells revealed that both HTLV-1/SU2 and HTLV-1/TM2 recombinant viruses shifted the cellular tropism of wtHTLV-1 from CD4+ T cell predominance to CD8+ T cell predominance, which was comparable to wtHTLV-2. Thus, our data indicates that although only SU binds to the cellular receptor, both SU and TM contribute to the transformation tropism. Our results in the context of the provirus reiterates the in vitro binding studies indicating that the HTLV-1 SU component is responsible for the preferential binding to CD4+ T cells. This implicates that the transformation tropism of HTLV-1 is indeed dictated at the receptor binding stage. Our data also suggests that TM component plays a significant role in conferring this tropism. Whether TM complements SU at the receptor binding stage to confer tropism or, there are additional determinants at the post-entry level conferring this tropism irrespective of the role of SU is yet to be explored.

A NOVEL ANTIVIRAL STRATEGY OF STIMULATING INTEGRASE'S NONSPECIFIC NUCLEASE ACTIVITY TO DAMAGE RETROVIRAL DNA

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In addition to activities needed to catalyze integration, retroviral integrases have a nonspecific endonuclease activity (J Virol 70:2598, 1996) that is enhanced by certain small compounds (J Biol Chem 276:114, 2001). These facts suggest a novel antiviral strategy in which integrase is stimulated to damage viral DNA before integration occurs (with any damage to cellular DNA being limited to cells that had just been entered by the virus and thus also blocking infection). In fact, we have found that noncytotoxic concentrations of some integrase stimulator (IS) compounds inhibit the replication of Rous sarcoma virus and human immunodeficiency virus. To identify additional IS compounds that induce integrase to nick DNA nonspecifically, we obtained support from the NIH Microbicide Innovation Program (R21AI075929) and developed a nonradioactive assay that is suitable for high-throughput chemical screening (Anal Biochem 396:223, 2010). The assay uses a 49-mer oligonucleotide that is 5'-labeled with a fluorophore, 3'-tagged with a quencher, and designed to form a hairpin that mimics the radioactive double-stranded substrates in gel-based nicking assays. Reactions are conducted in 384-well plates, then analyzed on a real-time PCR machine after a single heat denaturation and subsequent cooling to a point between the melting temperatures of unnicked substrate and nicked products (no cycling is required). Under these conditions, unnicked DNA reforms the hairpin and quenches fluorescence, whereas completely nicked DNA yields a large signal. The assay was linear with time, stimulator concentration, and amount of integrase, and 20% concentrations of dimethylsulfoxide (the solvent for many chemical libraries) did not interfere with the results; it also had an excellent Z'-factor and reliably detected known IS compounds. We are currently using this assay to screen a library of 50,000 drug-like chemicals to identify additional IS compounds for the novel antiviral strategy of stimulating integrase to destroy retroviral DNA.

RESTRICTION AND HYPERMUTATION OF THE AKV MURINE LEUKEMIA VIRUS BY MOUSE APOBEC3

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Background: APOBEC3 proteins are cytidine deaminases that can provide intrinsic immunity against a broad range viruses and retrotransposons. The best-characterized member of this enzyme family is human APOBEC3G, which can restrict the infectivity of Vif-deficient HIV and introduce harmful hypermutations in the single-stranded DNA replication intermediates of the virus. Endogenous mouse APOBEC3 has been shown to restrict murine retroviruses such as Friend MLV and the mouse mammary tumour virus, but in absence of detectable levels of deamination. AKV is an endogenous leukemogenic retrovirus of the AKR mouse strain. In this study, we investigate whether sensitivity of AKR mice to AKV could in part be explained by a failure of the APOBEC3 allele expressed in these mice to restrict AKV.

Materials and methods: *In vitro* assays were performed by infecting NIH 3T3 cells with viruses produced in 293T cells transiently expressing APOBEC3 proteins. *Ex vivo* infection assays were performed by infecting lymphocytes isolated from the spleens of various mouse strains or from those of APOBEC3-deficent mice. FACS analysis was used to measure the infectivity of eGFP-expressing indicator viruses in NIH 3T3 target cells.

Results: Here we show that endogenous APOBEC3 can inhibit the infectivity of the AKV murine leukemia virus, and that this restriction is associated with hypermutations in the retroviral genome that are attributable to the deaminase activity of APOBEC3. This restriction, however, is highly dependent on the level of APOBEC3 mRNA expression which appears to be particularly low in AKR mice and polymorphic amongst various mouse strains.

Conclusions: Our findings demonstrate that APOBEC3 confers an effective protection to mouse lymphocytes against retroviral pathogens when expressed at sufficient levels, and also provide good support to the proposal that APOBEC3-mediated hypermutation may have contributed to murine retroviral genome evolution.

HIV-1 RT MEDIATED MUTATION SYNTHESIS DURING DNA POLYMERIZATION IN MACROPHAGE NUCLEOTIDE POOLS

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HIV-1 reverse transcription is an essential step in the HIV-1 viral lifecycle, and both its efficiency and fidelity are directly dependent upon the cellular 2'-deoxy ribonucleotide triphosphate (dNTP) concentration. Reverse Transcriptase (RT) exhibits high dNTP binding affinity which facilitates replication in macrophages with low dNTP pools, and are thought to be a potential reservoir in the host. Moreover HIV-RT is an error prone polymerase relative to those responsible for both bacterial and eukaryotic genome replication, and is thought to be a prominent driver of viral mutagenesis resulting in the viral diversity required for drug resistance and immune evasion.

Here, we hypothesize that the HIV-1 replication is more error-prone in macrophage than activated CD4 T cells due to: i) extremely low dNTP concentration in macrophage, which induces RT pausing known to generate mutational hot spots, ii) imbalanced dNTP pools, and iii) higher incorporation of ribonucleotide triphosphates (rNTPs) as a consequence of rNTP/dNTP ratio in macrophage relative to known RT kinetic properties. First, we have measured the dNTP and rNTP pools with LC/MSMS in both cell types, which facilitates direct simulation of biologically relevant substrate conditions in all biochemical experiments. Second, using the estimated cellular nucleotide concentrations, we found that HIV-1 RT synthesizes more mutations in macrophage dNTP pools than in the activated CD4 T cell dNTP pools with a M13 lacZα forward mutation assay. Interestingly, the macrophage dNTP pools generate fourfold more point mutations than frame-shift mutations, while the T cell dNTP pool produces a similar ratio between point mutations and frame shift mutations. Third, we observed that HIV-1 displays an equal incorporation efficiency of both dNTP and rNTP at their concentrations found in macrophages. We are currently investigating the impact of rNTP incorporation on DNA polymerization kinetics and mutation synthesis both biochemically with appropriate cellular substrate pools and in biologically relevant primary human cells. Study of mutation rate in HIV-1 target cells will provide valuable insight into viral diversity generation, replication, and persistence in the host.

TOWARDS A TRANSGENIC RABBIT MODEL OF HIV-1 INFECTION

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An immunocompetent, permissive small-animal model would be valuable for the study of HIV-1 pathogenesis and for the testing of drug and vaccine candidates. However, the development of such a model has been hampered by the inability of primary rodent cells to efficiently support several steps of the HIV-1 replication cycle. Although transgenesis of the HIV receptor complex and human Cyclin T1 have been beneficial, additional late-phase blocks prevent robust replication of HIV-1 in rodents and limit the range of in vivo applications. In this study, we explored the HIV-1 susceptibility of primary T-cells and macrophages derived from New Zealand white rabbits. Envelope-specific and co-receptor-dependent entry of HIV-1 was achieved by expressing human CD4 and CCR5. A block at HIV-1 DNA synthesis, likely mediated by rabbit TRIM5, was overcome by limited changes to the HIV-1 gag gene. Unexpectedly and in stark contrast to mice and rats, primary cells from rabbits fully supported the functions of the regulatory viral proteins Tat and Rev, Gag processing, and the release of HIV-1 particles at levels comparable to human cells. While HIV-1 produced by rabbit T-cells was highly infectious, a macrophage-specific infectivity defect was identified that became manifest by a novel pattern of hypermutations. These results reveal an extraordinary natural HIV-1 permissivity of the rabbit species. Receptor complex transgenesis combined with modifications in gag and possibly vif to evade species-specific restriction factors might render lagomorphs fully permissive to infection by this pathogenic human lentivirus.

DELINEATING THE ROLE OF ADAPTOR PROTEIN COMPLEXES DURING HIV-1 PRODUCTION IN PRIMARY HUMAN MACROPHAGES.

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Previous studies indicate that the HIV-1 structural protein Gag drives virus assembly, and its expression alone is sufficient to form virus like particles. However, current understanding of the mechanism(s) by which Gag localizes to the site of virus assembly is limited. In primary T-cells, T-cell lines, as well as epithelial cell lines that support viral replication, virus assembly is thought to occur at the plasma membrane. Alternatively, in primary human macrophages, virus assembly has been reported to occur on late endosomes as well as at the plasma membrane. Additional studies indicate that Gag co-localizes with late endosomes in a variety of cell types, regardless of the intracellular site of virus assembly, which suggests a functional role for late endosomes in the viral replication cycle.

Adaptor protein (AP) complexes 1 and 3 have been reported to mediate Gag trafficking to late endosomes through a direct interaction of their subunits μ and δ, respectively, with the matrix region of Gag. Disruption of the AP-1μ/Gag or AP-3δ/Gag interactions has been shown to reduce Gag co-localization with late endosomes and lead to a block in particle production. Taken together, these results strongly suggest that AP-1 and AP-3 play key roles in the late stage of viral replication. However, the role of AP-1 or AP-3 in HIV-1 replication has not been demonstrated in natural host cells or in cells that support virus assembly on late endosomes. In this study, we ablate endogenous AP-1μ or AP-3δ function using RNA interference, and analyze HIV-1 replication and the intracellular localization of Gag in primary human macrophages.

THE ROLE OF TRIM5A RING DOMAIN WITH E3 UBIQUITIN LIGASE ACTIVITY IN RETROVIRUS RESTRICTION

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Upon entry into the host cell, retrovirus undergoes uncoating of the viral casid and completes generation of reverse-transcripts to form the pre-integration complex (PIC), which delivers retroviral reverse transcipts into the host genome. The host cellular factor, TRIM5α, was discovered to block retroviral infection in species-specific manner. How TRIM5α blocks the infection is a still unsolved question.
As a member of the tripartite motif (TRIM) family of proteins, TRIM5α contains RING, B-box, coiled coil, and B30.2 domain. Each of these domains has its own function(s) contributing to the ability of TRIM5α to block retrovirus infection. B30.2 domain has been suggested to recognize the incoming sensitive viral capsid. The coiled coil domain is responsible for multimerization to form trimer. B-box also has been reported to be essential for retroviral restriction. The N-terminal RING domain has E3 Ubiquitin ligase activity. However the precise role of the RING domain that plays in retroviral restriction has not been addressed. Recently, effect of proteasome inhibitor on HIV-1 infection was reported, suggestting that ubiquitin-related pathways might be involved in retroviral restriction. In an effort to address what functions TRIM5α RING domain has and how these functions are connected to viral restriction, we found that one amino acid change within RING domain switched on and off the restriction ability of some TRIM5α against HIV-1/SIV without affecting functions mediated by other domains. By using series of mutants within the RING domain, chimera proteins, and other biochemical assays, we investigated the relationship between HIV-1/SIV restriction and in vitro E3 ligase activity mediated by RING domain. Several evidences not only confirm some of effects of proteasome inhibitors on viral restriction, but also extend further our understandings on how TRIM5a blocks retroviral infection.

CHARACTERIZATION OF SPECIES-SPECIFIC INTERACTION BETWEEN BST-2 TRANSMEMBRANE DOMAIN AND HIV-1 VPU IN LIPID BILAYERS OF LIVING CELLS

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Bst-2, also known as tetherin, is an interferon-inducible antiviral host factor that blocks the release of HIV and other retroviruses. HIV-1 Vpu antagonizes Bst-2 via inducing its β-TrCP-dependent degradation and/or sequestration. Vpu antagonizes only against human Bst-2 (huBst-2), but not Bst-2 proteins derived from other species such as African green monkey Bst-2 (agmBst-2). It was previously reported that the transmembrane domain (TM) of Bst-2 defines this species-specific antagonism. However, it remains unclear how Vpu recognizes the TM domain of huBst-2. In this study, we performed a series of mutagenesis analysis to determine the region in Bst-2 which is responsible for its species-specific interaction to Vpu. We developed a novel technique based on bi-molecular fluorescence complementation (BiFC). The amino- and carboxy- terminal fragments of Kusabira-Green fluorescent protein (KGN and KGC) were individually fused to Bst-2 and codon optimized Vpu proteins. Bst-2 and Vpu interaction facilitated association of the KG fragments to produce a stable fluorophore. Using KGN-Vpu-stably expressing HEK 293 cells, we were able to quantify the amount of Bst-2 and Vpu complex in live cells. A series of KGC-tagged plasmids encoding mouse Bst-2-based chimeras with substitutions involving corresponding domains from reciprocal huBst-2 were transfected into the KGN-Vpu-expressing cells. As expected, mouse Bst-2-based chimeras only replaced with the reciprocal TM of huBst-2 significantly provided a KG fluorophore signal. We further performed alanine-scanning mutagenesis within the huBst-2 TM. The results led to the identification of several a.a. residues as interaction motif to Vpu. Interestingly, this motif was highly conserved among primate Bst-2, although the ability of agmBst-2 to interact Vpu was clearly lower than that of huBst-2. We hypothesized that this difference of Vpu interaction efficiency is due to the interface change within α-helix structure of TM domain by the two a.a. insertion. We generated the a.a.-inserted agmBst-2 mutant (agmBst-2 LL) and analyzed the Vpu interaction to detect the effects of the insertion. The ability of agmBst-2 LL to interact Vpu was significantly augmented compared to that of agmBst-2, suggesting that the presence of two amino acids is critical for both specific interaction and as a space holder. These data implicates that Vpu is specifically adapted to interact huBst-2 by recognizing its TM sequences and contributes to the efficient replication of HIV-1 in human cells.

STRUCTURAL INSIGHTS INTO CO-EVOLUTION OF THE P1-P6 CLEAVAGE SITE AND NELFINAVIR-RESISTANT HIV-1 PROTEASE.

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Background. The evolution of resistance against anti-retroviral therapy is a major obstacle in the treatment of HIV-1 infection. HIV-1 protease is an important therapeutic target as its function is essential for viral maturation. However, the selective pressure of protease inhibitor therapy results in a wide spectrum of mutations that confer resistance to nearly all drugs in this class. Furthermore, the virus also evolves mutations within the Gag polyprotein, which sometimes correlate with known sites of resistance in the protease. Previously we showed that Gag L449F and S451N mutations within p1-p6 cleavage site of Gag co-evolve with the nelfinavir-resistant D30N/N88D protease variant. We present here structural changes resulting from this co-evolution.
Methods. In this study, X-ray crystallography was used to investigate in detail the structural changes resulting from co-evolution of p1-p6 cleavage site. Structures of WT and nelfinavir-resistant protease in complex with p1-p6 peptide variants were determined and compared to WT protease in complex with the WT p1-p6 peptide.
Results. In the WT protease, Asp30 interacts with Arg 452 within the p1-p6 cleavage site. The D30N mutation disrupts this interaction and causes arginine to adopt new orientation. However, this loss of interaction is compensated by the co-evolving L449F and S451N mutations in the p1p6 cleavage site, which significantly increase available surface area for interaction with the protease. Increased interactions were also observed between Asp 29 and Ile 50 of the protease and the p1-p6 peptide.
Conclusions. These results suggest that the reduced interactions with p1-p6 peptide resulting from D30N/N88D protease mutations are partly compensated by additional changes within this cleavage site. These findings highlight the interdependency of interactions between HIV-1 protease and the p1-p6 substrate. In conclusion, this study provides a rationale for substrate co-evolution and the impact this has on resistance to anti-retroviral therapy.

PRODUCTION OF GFP-INCORPORATED INFECTIOUS PSEUDOVIRION BY THE N-TERMINAL MODIFICATION OF HIV-1 GAG

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An infectious HIV-1 virion incorporating a fluorescent marker allows us to visualize the viral entry in living cells. To generate a high titer infectious virus containing more than a thousand GFP molecules per virion, we genetically engineered amino-terminus of HIV-1 Gag.

In a previous study, we showed that substitution of the HIV-1 Gag myristoylation signal with the phospholipase C (PLC)-delta 1 pleckstrin homology (PH) domain or attachment of heterologous myristoylation signals on the amino-terminus of Gag increased the production of the HIV-1-based lentiviral vector (Urano et al, J Gen Virol 2008; Aoki et al, in submission). The infectivity of these pseudovirions was comparable to that of the wild type. These data suggest that the addition of foreign proteins at the amino-terminus of Gag do not damage the viral infectivity. We therefore tested whether the attachment of GFP at the amino-terminus of Gag is able to produce infectious pseudovirion.

We constructed two mammalian gag-pol expression plasmids, myr-GFP-gag-pol and GFP-PH-gag-pol. The former has lyn myristoylation signal attached to the amino-terminus of GFP-gag. The latter has GFP fused to the PH-gag-pol construct (Urano et al, J Gen Virol 2008). The gag-pol is human codon-optimized, and the original start codon of Gag is destroyed. Expressed in 293T cells, the majority of the mutant Gag/Gag-pol proteins were distributed to the plasma membrane similar to Gag-GFP as judged by the GFP fluorescence. The VLP production by the mutant gag-pol was verified by Western blotting, and visualized under the confocal microscopy. The efficiency of VLP prduction by the mutants was reduced by 2-fold compared with the WT. The pseudovirions produced by these mutants were infectious. The infectivity of mutant viruses relative to the WT is under evaluation. The proviral construction bearing similar genetic modifications is underway. The high titer "bright" virions containing approximately 3,000 GFP molecules per virion should be useful in single virus tracking studies.

MUTATIONS IN THE MEMBRANE-SPANNING DOMAIN OF HIV-1 GP41 CAUSE A KINETIC DEFECT IN THE MEMBRANE FUSION

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Many mutations in the highly conserved membrane-spanning domain (MSD) of the HIV-1 gp41 negatively affect the efficiency of membrane fusion. However, the exact mechanism is not well understood. In this study, we applied a novel cell-based fusion assay to analyze mutants of gp41 MSD. The new assay allows us to perform a real-time kinetic assay of membrane fusion in a simple manner. This system relies on the recovery of the functions of a pair of split proteins composed of split Renilla luciferase (Rluc) and split green florescent protein (GFP). These reporters recover both RL and GFP functions upon reassociation and hence named dual split proteins (DSPs). One of DSPs was expressed in 293FT cells together with HIV-1 Env, and the other was expressed in 293CD4 cells. These cells were cocultured to induce membrane fusion and the process was monitored continuously. Several gp41 mutants were analyzed: some of which have the entire MSD replaced with heterologous MSDs and others carry point mutations of the conserved arginine residue in the middle of the MSD. All these mutants showed slower kinetics of membrane fusion as compared with the wild type. This tendency was more prominent in the mutants carrying heterologous MSDs. The preliminary data suggest that these mutants have the defect in conformational changes during membrane fusion. Thus, the mutations within the MSD seem to cause an allosteric negative effect on the extracellular portion of HIV-1 Env.

A CONSERVED EXXXLV-BASED SORTING SIGNAL IN THE VPU CYTOPLASMIC TAIL IS REQUIRED FOR TETHERIN ANTAGONISM AND CELL-SURFACE DOWNREGULATION

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The HIV-1 accessory protein Vpu antagonizes the restriction of retroviral particle release by Tetherin (CD317/BST2) and mediates its cell surface removal and degradation. Vpu localizes to multiple membrane compartments, including the Trans-Golgi Network (TGN). Recent evidence suggests that TGN localization of Vpu is important for tetherin antagonism, but the subcellular trafficking of Vpu is undefined. Addition of an endoplasmic reticulum (ER) retention signal (KKDQ) to the C-terminus of Vpu blocked its ability to antagonize tetherin-mediated restriction, implying that post-ER trafficking of Vpu is essential for its function. We found that Vpu localizes to compartments that contain the cation-independent mannose-6-phosphate receptor (CI-MPR), suggesting a dynamic trafficking of Vpu between TGN and endosomal compartments. Consistent with this, we have identified a conserved variant of an acidic/di-leucine sorting signal, ExxxLV, in the second alpha-helix of the Vpu cytoplasmic tail. Targeted disruption of this sequence in the HIV-1 NL4.3 Vpu protein led to its accumulation in endosomal compartments. Furthermore, mutation of the ExxxLV motif impaired the ability of Vpu both to rescue HIV-1 particle release in the presence of tetherin, and also to downregulate cell-surface tetherin levels. Interestingly, in the presence of wildtype Vpu, tetherin itself localizes predominantly to the TGN. Since D/ExxxL(L/I/V) sorting motifs are known to interact with multiple clathrin adaptors to mediate trafficking of membrane proteins between the endosome and TGN, these data indicate that the ability of Vpu to traffic between these compartments is required for its anti-tetherin activity, potentially to block tetherin recycling to the plasma membrane. The roles of cellular factors known to be required for these processes are currently being investigated.

ENV AND VPU-DEPENDENT RESCUE OF HIV-1 P6 ALIX BINDING SITE MUTANTS.

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The HIV-1 Gag polyprotein precursor is responsible for directing virus assembly and release, and contains domains that mediate specific steps in the pathway of virus production. The p6 domain of HIV-1 Gag is required for virus budding. Small peptide motifs in p6, known as “late” or “L” domains, promote the release of virions from infected cells by interacting directly with host cell factors. Specifically, a Pro-Thr-Ala-Pro (PTAP) motif binds the ESCRT-I component Tsg101 and a Tyr-Pro-Xn-Leu (YPXnL, where X is a variable residue and n is 1–3) motif binds Alix, a protein that interacts with both ESCRT-I and ESCRT-III. The primary Alix binding sequence is located near the C-terminus of p6, where p6-Y36, L41, and L44 are particularly critical for Gag–Alix binding. Previous work from our lab has demonstrated a functional role of p6–Alix binding in HIV-1 particle production and replication. Specifically, mutations were introduced at residues Y36, L41 and L44 in p6 to characterize the effects on 1) binding to the Alix-V domain, 2) HIV-1 Gag processing and virus release, 3) replication kinetics in a variety of relevant cell types, 4) virion morphogenesis and 5) single-cycle infectivity. One of the most striking results was the severe delay in virus replication in Jurkat T cells induced by these mutations. Viruses collected at the delayed replication peaks demonstrated enhanced replication kinetics upon re-passaging to fresh Jurkat T cells relative to the original mutants. Sequencing of the putative revertant isolates revealed several mutations mapping to gp120, gp41 and Vpu. We have confirmed that the Env and Vpu mutations do indeed compensate for the effects of the p6 mutations on virus replication, and have characterized the effects of the Env and Vpu mutations on HIV-1 Gag processing and virus release in HeLa cells. We have also examined the effect of these compensatory mutations in single-cycle infectivity, cell-free infection and cell-to-cell infection of relevant cell types. We propose that defects in cell-to-cell transmission induced by mutations in the p6 Alix binding site are rescued by the substitutions in Env and Vpu.

EARLY EVENTS IN HIV-1 ASSEMBLY AND RNA PACKAGING

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HIV-1 Gag polyprotein is the only viral protein that is required for particle assembly and drives a number of different events during this process. Although we have a reasonable understanding of the molecular details of events that take place at the late stages of HIV assembly, the early assembly processes mediated by Gag are still not well defined. In this regard, an important question is where in the cell Gag initially interacts with the viral RNA. A second key question is whether Gag-RNA interaction requires or induces higher order Gag multimerization in cells. To address the first question, we developed an assay in which RNA-immunoprecipitation was coupled with membrane flotation analysis in transfected or infected cells. We found that Gag-RNA interaction takes place in the cytoplasm, is independent of the ability of Gag to localize to the plasma membrane, but was influenced by the C-terminal domain (CTD) of capsid (CA), which mediates multimerization of Gag in virions. Specifically, the absence of the CA CTD does not lead to a defect in recruitment of viral RNA to the plasma membrane. However, the formation of a stable immunoprecipitable complex containing Gag and viral RNA at the plasma membrane required intact CA.

In an attempt to address the second question, we employed a cross-linking based Gag multimerization assay, coupled to membrane flotation analyses. These assays indicated that Gag is present as monomers and low order multimers in the cytoplasm, but does not form higher-order multimers in the cytoplasm. Rather, higher order multimers formed only in the plasma membrane fractions, and when Gag was able to bind to membrane. Taken together, our results suggest that the initial site of Gag-RNA interaction is the cytoplasm, involves Gag monomers or low order multimers and that these interactions per se do not induce or require higher order Gag multimerization in the cytoplasm. Instead, membrane interactions appear to be required to induce higher order Gag multimerization in cells.

ANALYSIS OF THE INTERACTION OF WILD-TYPE AND MUTANT HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 PROTEASE WITH HIV-1 PROTEASE INHIBITORS IN SILICO AND IN VITRO

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Human T cell leukemia virus type 1 (HTLV-1) has infected >30 million individuals worldwide, of which up to 3-5% will develop an aggressive and terminal adult T-cell leukemia for which there is currently no effective treatment. The HTLV-1 protease has been reported to be resistant to all commercial HIV-1 protease inhibitors. And while HTLV-1 infections can be treated with HIV-1 reverse transcriptase inhibitors, resistance has developed. The X-ray crystal structure of HTLV-1 protease with a bound peptidomimetic inhibitor was solved. Using this structure, the interaction of the HTLV-1 protease with commercial HIV-1 protease inhibitors was analyzed using computational chemistry software. The interaction energy scores between the wild type and mutant HTLV-1 proteases and commercial HIV-1 protease inhibitors were calculated and revealed that both proteases bound with a similar strength to the inhibitors, using structurally equivalent residues/atoms. The HTLV-1 PR was then mutated to test whether binding to HIV-1 PR inhibitors could be increased. An in vitro fluorescent assay was then used to quantitate the wild-type and mutant HTLV-1 PRs binding to HIV-1 inhibitors. This work is directed at understanding how HTLV-1 protease interacts with native substrates and HIV-1 protease inhibitors, so that effective therapies can be developed to target the HTLV-1 PR and control HTLV-1 infections.

IDENTIFICATION OF AN APOBEC3 RESIDUE THAT SEPARATES CELLULAR LOCALIZATION FROM HIV-1 RESTRICTION

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APOBEC3B and APOBEC3G are both double domain cytidine deaminases, sharing 68% amino acid similarity. APOBEC3G is a cytoplasmic protein with potent HIV-1 restrictive ability, while APOBEC3B predominantly resides in the nucleus and is a comparatively weak restrictor of HIV-1. Current models suggest that cytoplasmic APOBEC3G is packaged into assembling HIV-1 particles en route to infection of an unsuspecting target cell where retrovirus restriction by cytidine deamination occurs during reverse transcription. Thus, it is tempting to hypothesize that cellular localization is a property that determines which APOBEC3 can restrict HIV-1. Here, we identify a residue in APOBEC3B that when mutated to match its counterpart in APOBEC3G, allows APOBEC3B to become a potent HIV-1 restrictor. Surprisingly, this APOBEC3B variant retains nuclear localization. These data suggest that an as yet unidentified mechanism exists to target the APOBEC3 proteins to their retroviral substrates. These results have implications for endeavors to boost the activity and/or expression of these potent deaminators for anti-viral therapeutic purposes.

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STIMULATION OF AP1 AND NFKB SIGNAL TRANSDUCTION PATHWAYS BY TRIM5

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The human Trim5 alpha protein possesses a capsid-specific restriction activity against a broad range of retroviruses, including a small effect on HIV-1. TRIM5 mRNA is up-regulated upon Type I IFN stimulation. This fact, along with other observations, stimulated a search for other links between TRIM5 and innate immune signal transduction. TRIM5 knockdown was found to decrease the antiviral effect of LPS and other pathogen recognition receptor (PRR) agonists. Then the effect of TRIM5 on the activity of luciferase reporters for transcription factors involved in innate immunity was tested. TRIM5 overexpression had a modest stimulatory effect on NFkB (<5-fold) but a very strong induction of AP-1 reporter (50 to 150-fold). The activity of different TRIM5 orthologues and a panel of TRIM5 mutants on AP1 reporter induction was tested next. The ability to induce the NfKB and AP1 reporters was conserved in TRIM5 orthologues from macaques (Macaca mulatta), two species of african green monkey (Cercopithecus), and even the TRIM5-cyclophilin A fusion proteins of owl monkey (genus, Aotus) and macaque. In fact, the owl monkey fusion protein was ~5 times more potent than human Trim5 alpha. In future experiments we will map domains critical for these activities.

CO-ACTIVATOR FUNCTION OF SAP18-HDAC1 COMPLEX IN INI1/hSNF5-MEDIATED ACTIVATION OF INTERFERON-STIMULATED GENES

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HDAC1 complex deacetylates histones to mediate transcriptional repression. However, HDAC1 complex also activates transcription of certain genes (e.g. interferon signal induced genes, ISGs) by deacetylating non-histone transcription factors (Jana et al Blood 2007; Nusinzon et al Science 2005). INI1/hSNF5, an HIV-1 IN binding protein, directly interacts with SAP18 (Sin3a associated protein 18kD), a component of the Sin3a-HDAC1 complex and selectively recruits it to HIV-1 virions (Sorin/Cano et al PLoS pathogens 2009). INI1 transcriptionally activates ISGs including IFIT1, IFITM1, IFITM3, ISG15, PML and OAS etc. in INI1-/- cells (Morozov/Lee et al Clin. Can. Res. 2007).
To determine the significance of INI1-associated SAP18-HDAC1 complex to interferon signaling, we isolated a panel SAP18 interaction defective mutants of INI1 (SID-INI1*), and tested them for ability to stimulate ISGs. We found that unlike wild type INI1, SID-INI1* were defective for activation of ISGs. In addition, we found that: (i) TSA (histone deacetylase inhibitor); (ii) siRNA to HDAC1; and (iii) expression of a dominant negative mutant of HDAC1 (HDAC1H141A) all inhibited INI1-medaited activation of ISGs. Furthermore, the addition of HDAC1 stimulated the INI1-mediated activation of ISGs. Chromatin-immunoprecipitation assay indicated that INI1 and SAP18 directly bind to IFITM1 promoter. SID-INI1* mutants, while being able to bind to IFITM1 promoter, were unable to recruit SAP18 and HDAC1, indicating that interaction of INI1 with SAP18 is required for recruitment of HDAC1 complex to ISG promoter. These results, together, indicate that SAP18-HDAC1 complex is necessary for INI1-mediated induction of ISGs and provide direct evidence for co-activator function of SAP18-HDAC1 complex.
We have previously established that binding of IN to INI1 leads to encapsidation of INI1 associated SAP18-HDAC1 complex into HIV-1 virions. We propose that depletion (and/or inactivation) of this complex in the producer cells, by IN, may inhibit interferon signaling. However, the knock-down of INI1 in target cells may lead to down-modulation of interferon signaling, and thus increased HIV-1 replication.

EFFECTS OF B -ESTRADIOL ON THE SAFETY OF ANTI-HIV DRUGS

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Background: Female hormones are known to play an important role in predisposition for many infectious diseases. Epidemiological study suggests there are gender effects in serum HIV-1 load.

Objectives: To determine whether β-estradiol affects the replication of HIV-1 or the efficacy of a common anti-retroviral drug, Stavudine (D4T). To determine the effect of β -estradiol on metabolic conversion of D4T into active drug D4T-TP and critical enzymes involved in D4T metabolism.

Methods: Human PBL from different donors or various T cell lines were infected with HIV-1 in the presence or absence of combinations of β-estradiol and the anti-retroviral drug, D4T. After four and seven days in culture, supernatants were assayed for HIV-1 p24 protein. H9 cells were treated with radiolabeled (3H) D4T or radiolabeled (3H) thymidine in the presence and absence of β -estradiol and extracted at various time points to follow the metabolic conversion of D4T into the active metabolite and thymidine kinase activity.

Results:. β-estradiol resulted in a modest inhibition of HIV-1 replication in male- and older-female-derived PBLs, but β-estradiol did not affect the HIV-1 P24 level in PBLs from female donors, suggesting that β-estradiol attenuates the anti-HIV-1 efficacy of D4T in primary PBLs in a gender- and age-specific manner.
β-estradiol also inhibited HIV-1 replication and attenuated the anti-HIV-1 efficacy of D4T in H9 cell lines, facilitating studies of mechanism. Results will be presented on the effects of β -estradiol on the level of the active drug metabolite D4T-TP and on the activity of thymidine kinase, which phosphorylates D4T to D4T-P.

Conclusion: β -estradiol impairs the antiretroviral efficacy of D4T through affecting drug metabolism. To optimize antiretroviral drug therapy, it may be necessary to monitor patient hormonal status.

POLYMORPHISMS IN HUMAN APOBEC3H DETERMINE ITS SENSITIVITY TO VIF AND SUBCELLULAR LOCALIZATION: IMPLICATIONS FOR HIV-1 EVOLUTION

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The APOBEC3 family of cytidine deaminases (APOBEC3A-H) restricts various retroviruses and retroelements by causing G-to-A hypermutation in the viral genome and other mechanisms. In order to replicate efficiently in vivo, retroviruses have evolved strategies to evade or counteract APOBEC3 antiviral activities. HIV-1 encodes the viral Vif protein that binds and targets APOBEC3 for degradation. However, it is not completely clear how Vif counteracts multiple APOBEC3 proteins at once.

Previous work in the lab has shown that APOBEC3H is polymorphic in humans, with four major population-dependent haplotypes that encode proteins with different levels of antiviral activity. Haplotype II, present most frequently in African populations, encodes the most stable protein and is most active against HIV-1. We recently showed that this APOBEC3H protein is mostly resistant to HIV-1 LAI Vif. In order to find Vif variants that can better counteract APOBEC3H, we characterized the evolution of vif alleles within individuals carrying haplotype II over the course of infection and found at least one individual where APOBEC3H is a driving force of Vif evolution. We mapped the Vif determinant important for functional interaction with APOBEC3H that is independent of Vif counteraction of APOBEC3G and 3F. Using recently developed monoclonal antibodies against endogenous APOBEC3H, we are investigating the tissue expression profile of APOBEC3H in individuals who are likely to encode the stable protein.

Surprisingly, one of the polymorphisms in APOBEC3H that determines the stability of the protein is also responsible for the differential subcellular localization of the various human APOBEC3H proteins. We found that haplotype II encodes a cytoplasmic protein whereas the less active protein encoded by haplotype I is localized to both the cytoplasm and nucleus which could be potentially more mutagenic to host genome. The relevance of APOBEC3H localization to function is currently being explored.

BIOCHEMICAL AND BIOPHYSICAL ANALYSIS OF HIV-1 INTEGRASE PROTEINS DERIVED FROM CLASS II MUTANT VIRUSES

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Integrase, the HIV-1 enzyme that catalyzes integration, has been implicated to play other roles in the viral lifecycle. Integrase directly interacts with reverse transcriptase and can affect the activity of recombinant polymerase *in vitro*. Integrase mutant viruses that are defective for HIV-1 replication at steps other than integration are typed as class II, and reductions in the level of viral DNA synthesis is the most common class II mutant viral phenotype. Integrase has also been shown to interact with various elements of the cellular nuclear import machinery and some integrase mutant viruses show marginal defects at viral cDNA nuclear transport. A third phenotype commonly associated with class II mutant integrase viruses is defective virus assembly and/or release from cells.

Integrase functions during integration as an obligate tetramer. Due to the relatively wide range of class II mutant phenotypes, we have investigated correlations between integrase multimerization, integrase activities, and the class II mutant phenotype. Eighteen integrase proteins derived from class II mutant viruses were purified following their expression in *Escherichia coli*. The purified proteins were assayed for integrase 3' processing and DNA strand transfer activities using relatively short oligonucleotide donor DNAs and denaturing polyacrylamide gel electrophoresis that does not distinguish single viral DNA end integration from concerted integration of two donor DNA ends. The mutants were also tested for concerted integration activity using longer restriction fragment donor DNA or short oligonucleotide donor DNA in the presence of the LEDGFp75 integrase-binding cofactor. Integrase multimerization was detected following protein cross-linking. The results of these experiments will be discussed.

CHARACTERIZATION OF APOBEC3G DNA DEAMINASE INHIBITORS IDENTIFIED BY HIGH THROUGHPUT SCREENING

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APOBEC3G (A3G) is a DNA cytidine deaminase that potently restricts HIV-1 and a variety of other retroelements. To identify small molecule inhibitors of A3G activity, we have developed, optimized and miniaturized a fluorescence-based single-strand DNA deaminase assay (1, 2). We used this assay to screen several small chemical libraries in collaboration with the Institute for Therapeutics Discovery and Development at the University of Minnesota. Thus far, we have evaluated approximately 6,000 compounds and we have identified 64 that appear to specifically inhibit A3G DNA deaminase activity. Kinetic, specificity, and NMR binding data indicate that low micromolar concentrations of these chemicals block activity by several distinct mechanisms. We will discuss these data and the results of cell-based studies currently in progress. We anticipate that these compounds will become useful chemical probes in the ongoing efforts to elucidate the A3G-dependent HIV-1 restriction mechanism.

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ANALYSIS OF PROTOTYPE FOAMY VIRUS GAG GLYCINE-ARGININE-RICH BOXES HIGHLIGHTS THEIR FUNCTIONAL EQUIVALENCE TO ORTHORETROVIRAL CYS-HIS MOTIFS

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Foamy viruses (FV) are complex retroviruses that display numerous unique features distinguishing them from all other retroviruses. FV Pol is expressed independently of Gag, and both Gag and Pol undergo only single cleavage events. Therefore, the mature FV Gag protein does not consist of the matrix, capsid, and nucleocapsid (NC) proteins as in orthoretroviruses. Furthermore the putative NC domain of FV Gag lacks the hallmark Cys-His motifs that are otherwise essential at different steps of the viral replication cycle, like reverse transcription, infectivity, RNA packaging, integration and viral assembly. However, FV Gag contains three glycine-arginine-rich boxes (GR boxes) instead that might resemble a functional equivalent to the Cys-His motifs. We used a transcriptional independent 4-Plasmid prototype FV (PFV) vector system to analyze deletion and substitution mutants of each GR box for particle release, particle protein composition, nucleic acid content, infectivity and intracellular localization. While viral particle release and particle protein composition are not affected by the mutations; DNA content and infectivity are severely reduced for deletion mutants and not detectable for full-length substitution mutants. Substitutions with shorter fragments then the original GR box show an intermediate phenotype. In addition GR box II mutants lack nuclear localization potential. Furthermore we show that the deficiency phenotypes are at least partially rescued by substitution of one GR box with another. Thus it appears that just like the Cys-His motifs in orthoretroviruses the PFV Gag GR boxes have numerous functions at different steps of the viral life cycle.

THE CYTOPLASMIC TAILS OF JSRV AND ENTV ENVELOPES ARE SUFFICIENT FOR ONCOGENIC CELL TRANSFORMATION

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Jaagsiekte sheep retovirus (JSRV) and enzootic nasal tumor virus (ENTV) are two closely related betaretroviruses that cause contagious lung and nasal tumors in sheep and goats, respectively. One unusual feature of these two acutely transforming retroviruses is that, in addition to mediating viral entry their envelope proteins also act as potent oncogenes eliciting oncogenic transformation in vitro and in vivo. We and others previously reported that the cytoplasmic tails (CTs) of JSRV and ENTV Env proteins are critical for cell transformation in vitro, but whether or not these domains are sufficient for oncogenesis remains elusive. Here we have generated a series of chimeric Envs between JSRV/ENTV and an endogenous sheep retrovirus that is not oncogenic, and determined their oncogenic potentials in vitro and in vivo. We found that, despite their relative low surface expressions in the transfected cells, chimeras harbouring the CTs of JSRV/ENTV Envs were able to induce substantial transformation in dog MDCK epithelial cells and rat 208F fibroblasts. Importantly, subcutaneous injection of the transformed MDCK cells into nude mice readily induced tumor formations. In addition, we found that the signalling pathways activated in the chimeras-transformed cells paralleled those of cells transformed by the wildtypes, further arguing that CTs of JSRV and ENTV Envs alone are sufficient for oncogenic transformation. While prior results from our lab and others showed that the YXXM motif in the CTs of JSRV and ENTV Envs are essential for cell transformation, here we have found that restoration of this motif into the endogenous JSRV Env was unable to induce transformation, implying that other regions of JSRV and ENTV CTs are also important. Indeed, using serial truncation mutants we were able to identify a dileucine motif in the C-terminus of JSRV Env CT to be critical for transformation. Structural analysis predicted that this dileucine motif is located at the immediate end of an extended alpha helix formed by the N-terminus of Env CT, separating it from the C-terminal random coils. Identification of cellular binding partners that directly interact with the JSRV and ENTV CTs will be crucial for fully understanding the mechanisms of JSRV/ENTV Env oncogenesis.

THE HIV-1 COFACTOR ACTIVITY OF LEDGF/P75 INVOLVES BOTH CHROMATIN TETHERING-DEPENDENT AND -INDEPENDENT MECHANISMS.

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The HIV-1 cellular cofactor LEDGF/p75 is required for efficient viral DNA integration. Analysis of the HIV-1 cofactor activity of LEDGF/p75 mutants supports a chromatin tethering model, indicating that LEDGF/p75 acts as a molecular tether that interacts with HIV-1 integrase targeting the HIV-1 pre-integration complex to the host chromatin. Our data have expanded this model by evidencing an active role of integrase during LEDGF/p75-mediated chromatin tethering as well as by demonstrating the implication of chromatin tethering-independent mechanisms in the HIV-1 cofactor activity of LEDGF/p75. We have observed that deletion of the PWWP domain (ΔPWWP) of LEDGF/p75 negligibly alters its HIV-1 cofactor activity despite decreasing its chromatin binding strength. A molecular explanation for these results comes from the analysis of the chromatin binding strength of the LEDGF/p75-integrase complex. Co-expression of LEDGF/p75 wild type or ΔPWWP with HIV-1 integrase increased the chromatin binding strength of these cellular proteins. In the case of LEDGF/p75 ΔPWWP, HIV-1 integrase rescued its chromatin binding strength to levels observed with the full-length protein. These evidences indicate that integrase can modulate the interaction of the LEDGF/p75-integrase complex with chromatin facilitating the HIV-1 cofactor activity of LEDGF/p75 ΔPWWP. In support of the chromatin tethering-independent mechanism of LEDGF/p75 in HIV-1 DNA integration, we have observed that mutations in the CR1 or CR3 regions of LEDGF/p75 decreased its HIV-1 cofactor activity without affecting the interaction of LEDGF/p75 with chromatin and/or HIV-1 integrase as evaluated by multiple assays. In summary, our data illustrate the complexity of the molecular mechanism of LEDGF/p75 in HIV-1 DNA integration.

THE VPU-MEDIATED RESTRICTION OF GIBBON APE LEUKEMIA VIRUS ENV WITH HIV-1 IS GAG DEPENDENT AND DISTINCT FROM CD4 DEGRADATION

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We recently found and reported that the HIV-1 accessory protein Vpu is responsible for the known pseudotyping restriction between HIV-1 and the gibbon ape leukemia virus (GaLV) Envelope (Env) protein. Here we characterize factors required for this newly described restriction by Vpu. First, we investigated whether GaLV Env restriction is Gag dependent. Because we previously observed restriction in the context of HIV-1 and not MLV particles, we determined the sensitivity of another lentivirus, simian immunodeficiency virus (SIV), and an additional gammaretrovirus, RD114. In the presence of Vpu, GaLV Env pseudotyping was restricted with SIV particles but not RD114 particles. Because the matrix (MA) region of Gag is thought to interact with Env, we next tested the sensitivity of an MLV Gag chimera that contained the MA domain from HIV-1. This construct (HIV-1-MA/MLV) displayed HIV-1 like sensitivity to Vpu in our GaLV Env model. These data suggest that Vpu restriction occurs in a Gag dependent manner and that the MA domain contributes to this restriction. Second, we sought to determine the minimal regions of Vpu required for the GaLV Env incompatibility. Based on a series of Vpu cytoplasmic truncations and mutations, we found that removal of the Vpu C-terminal alpha-helix restored infectivity in GaLV Env/HIV-1 particles. We then tested a Vpu serine mutant ($Vpu_{52/56}$) and a Vpu with a "scrambled" transmembrane domain (Vpu_{RD}) known to disrupt Vpu's antagonism of CD4 and tetherin (BST-2), respectively. In our GaLV Env model, we found Vpu_{RD}, but not $Vpu_{52/56}$, restricted infectious particle production. Previously we have shown that Vpu leads to a partial reduction in cellular GaLV Env protein, possibly the result of degradation. However, treatment of cells with either proteosomal or lysosomal inhibitors failed to restore the production of infectious particles, suggesting that degradation cannot fully explain incompatibility. However, like CD4, we found Vpu requires poly-ubiquitination for GaLV Env restriction, although it is unknown whether GaLV Env is directly poly-ubiquitinated. Collectively, our findings indicate that Vpu's mechanism for GaLV Env downmodulation appears to be distinct from the pathways previously described for modulation of CD4 and tetherin.

TARGETING THE PI3K/AKT CELL SURVIVAL PATHWAY WITH ALKYLPHOSPHOLIPID COMPOUNDS TO INDUCE CELL DEATH OF HIV-1 INFECTED MACROPHAGES

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HIV-1 infected macrophages and microglia are long-lived viral reservoirs that persistently produce viral progenies. We previously demonstrated that HIV-1 infection promotes the stress-induced activation of the PI3K/Akt cell survival pathway in primary human macrophages, which extends the life span of these cells. This led us to determine whether various Akt inhibitors could affect survival of HIV-1 infected cells. Here, we examined the effect of one such class of drugs, alkylphospholipids (ALPs), on apoptotic cell death in human macrophages and a human microglial subline, CHME5, transduced with HIV-1 vector. We also explored Akt cell survival signals in transduced CHME5 cells, as well as the effect of ALPs on HIV-1 production in human macrophages. Our findings revealed that the ALPs perifosine and edelfosine specifically induced death of HIV-1 infected primary human macrophages and CHME5 cells. Furthermore, these two compounds reduced phosphorylation of both Akt and GSK3β, a downstream substrate of Akt, in the transduced CHME5 cells. Additionally, we observed that perifosine effectively reduced viral production in HIV-1 infected primary human macrophages. These observations confirm that the ALP compounds tested are able to inhibit the action of the PI3K/Akt pathway, therefore promoting cell death in HIV-1 infected macrophages and CHME5 cells transduced with HIV-1 vector. Pharmacological treatment with ALPs in HIV-1 infected patients would introduce the possibility of reducing the survival capacity of macrophage and microglial reservoirs, consequently decreasing viral production and release.

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PROBING THE FUNCTION OF THE RSV SP ASSEMBLY DOMAIN BY IN VITRO ASSEMBLY OF GAG.

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Purified retroviral Gag protein can assemble *in vitro* to yield immature virus-like particles (VLPs). Despite little amino acid (AA) sequence identity in Gag, we show by electron cryo-tomography that the RSV Gag lattice is very similar to that previously reported for HIV-1 [1,2]. In both viruses, a prominent stalk-like density feature is apparent underneath the Gag hexamer, apparently formed by AAs just downstream of the folded CA-CTD. We showed previously that in RSV, a 24 AA segment including the spacer peptide (SP) is critical for immature virus particle assembly. [3]. Secondary structure algorithms predict that this SP assembly (SPA) domain is an amphipathic helix, similar to predictions made earlier for HIV-1 [4]. We hypothesize that the stalk-like density feature corresponds to the SP assembly domain and that it holds the Gag hexamer together.

To address this hypothesis, we have mutated individual AAs in the SPA domain to either Ala or Cys and assessed their role in *in vitro* assembly. The mutant proteins were expressed in *E. coli*, purified, and scored for their ability to assemble into VLPs. Mutations that abolish *in vitro* assembly are presumed to contribute to higher-order structures in VLPs. So far, the hydrophobic residues in the SPA domain do not tolerate mutations, but small, polar residues can be mutated without compromising assembly. These results are consistent with the hypothesis that the SPA domain forms a helix that interacts with adjacent SPA helices via hydrophobic interactions. We have modeled these interactions as a six-helix bundle like that in cucumber mosaic virus [5], as suggested originally by Wright *et al* [3]. Cysteine cross-linking and chemical modification experiments are in progress to test this model.

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FIV GAG CONTAINS A CONSENSUS CLATHRIN-BINDING MOTIF THAT IS REQUIRED FOR VIRUS REPLICATION AND INFECTIVITY

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We have recently shown that an LLDL sequence at the extreme C-terminus of FIV Gag is not required for virus release. However, we observe that FIV Gag LLDL mutants consistently fail to replicate efficiently in either feline kidney (CrFK) or T lymphocyte (MCH5-4) cell lines. Selection for second-site revertants has revealed mutations in FIV Gag and Env that may restore virus replication. VSV-G-pseudotyped FIV Gag LLDL mutants exhibit a profound defect in specific infectivity when VLPs are produced in CrFK or HeLa cells, but, curiously, this infectivity defect is lost if virus is produced from 293T cells. Although no significant difference in VLP production is observed when the LLDL sequence is mutated, levels of RT detected in virions are consistently 2 to 3-fold lower than those in wild-type VLPs. Interestingly, the LLDL sequence fits the consensus for a canonical clathrin-binding motif found in many other proteins, including the β subunit of clathrin-adaptor proteins (AP-1,-2,-3) and HIV proteins (Nef, Vpu). However, the ability of FIV Gag to bind clathrin has not yet been demonstrated. To determine whether clathrin may play a role in virus infectivity or possibly virus production, we have found that siRNA-mediated knockdown of clathrin heavy chain in HeLa cells inhibits both FIV and HIV-1 release efficiency by approximately 3-fold. Since the potential clathrin-binding LLDL motif of FIV Gag is not involved in virus release, this effect may rely on an unidentified clathrin-binding domain in FIV or HIV-1 Gag or is possibly an indirect effect. Thus, we propose that clathrin may be involved in FIV infectivity via the LLDL motif, and virus production in both FIV and HIV-1 through an unknown mechanism that is currently being defined.

SENSITIVITY OF CIRCULATING SIV ISOLATES TO TRIM5 PROTEINS

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Overall, at least 35 species of African nonhuman primate species are naturally infected with SIV, with a seroprevalence ranging from 10% to 60% in the wild. Thus, African monkey species is the largest SIV reservoir and could represent a potential risk for the introduction of new retroviral zoonoses into the exposed human population. Indeed, it is now clearly demonstrated that the HIV epidemic originated quite recently from zoonotic transmission of viruses from chimpanzees and sooty mangabeys in Africa. The host range of retroviruses is influenced by antiviral proteins such as TRIM5a, a restriction factor that recognizes and inactivates incoming retroviral capsids (CA).
Here, we wanted to investigate whether SIV (SIVgsn, SIVmus, SIVmon, SIVmnd1, SIVcol and SIVtal) found in primate bushmeat from Cameroon are sensitive to TRIM5 restriction.
For this purpose, we swapped the fragment of the capsid that spans the CypA binding loop in a gag-pol SIVmac expression vector with that of SIV isolates from the SIVgsn lineage (SIVmon, SIVgsn and SIVmus), SIVmnd1, SIVcol and SIVtal. Gag expression was determined by immunoblotting. Stable CHO cells expressing distinct TRIM5 were obtained by transduction with primate TRIM5 of different origins. We then evaluated by single-round infectivity assays the restriction of these CA chimeras into CHO cells and CHO cells stably expressing either different TRIM5a (human, chimpanzee, African green monkey, and rhesus macaque) or the owl monkey TRIM-Cyp. Infectivity was assayed by FACS 48 hours post-infection.

We observed that human and chimpanzee TRIM5a? did not restrict any of the SIV CA we tested. However, some SIV CA were restricted by agm-TRIM5a (SIVmon, SIVmnd-1) or slightly restricted by rh-TRIM5a (SIVgsn, SIVmon).

Interestingly, we found that SIVmnd1 and SIVgsn CA, and not SIVmon and SIVmus CA, were targeted by owl monkey TRIM-Cyp, suggesting that the interaction between cyclophilin A and lentiviral CA is not limited to HIV-1. On the other hand, TRIM5a does not seem to constitute a natural barrier to SIV infection of humans.

EXCESSIVE SPLICING OF HIV-1 RNA RESULTS IN DEFECTIVE GAG ASSEMBLY WHICH CAN BE COMPLEMENTED BY THE EXPRESSION OF PACKAGABLE VIRAL RNA

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HIV-1 RNA is alternatively spliced to produce completely spliced 1.8kb or incompletely spliced 4.0kb classes of RNA. Nearly half of the HIV-1 RNA remains as unspliced RNA, which is used to encode Gag and Gag-pol protein and to serve as genomic RNA (gRNA). HIV-1 RNA splicing is tightly regulated to optimize gene expression and efficient virus replication. Our laboratory has previously shown that HIV-1 mutations that cause excessive splicing of HIV-1 RNA results in a severe reduction in virion production and defective Gag processing. Studies with one of the oversplicing mutant by confocal microscopy have shown that Gag expressed by the wild-type virus (WT) is mainly associated with plasma membrane whereas Gag expressed by the oversplicing mutant is mainly cytoplasmic, suggesting that the excessive splicing causes the Gag assembly and membrane localization defect. Overexpression of Gag protein alone was unable to rescue the defect, but co-transfection of WT virus along with the mutant showed significant rescue of both mutant Gag processing and virion production. Deletion of packaging sequence/s from WT virus resulted in inefficient rescue of Gag processing by the oversplicing mutant virus. These results suggest that co-packaging of WT RNA is necessary to restore the Gag processing and assembly defect of the oversplicing mutant.
We have further shown that excessive splicing and Gag processing defect can be induced in wild-type HIV-1. Virus replication was inhibited using 5' end modified U1 snRNAs that bind strongly to weak 5'splice donor sites downstream of either 3'splice acceptor site (3'ss) A1 or A2. Splicing was also promoted by expression of modified U1 snRNAs targeted to conserved sequences downstream of 3' ssA3. Expression of modified U1 snRNAs to induce excessive splicing may have potential as a therapeutic approach for inhibition of virus replication.

NEF BOTH CONTRIBUTES TO THE ESTABLISHMENT OF HIV LATENCY AND ENHANCES THE REACTIVATION OF LATENT PROVIRUSES

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Many models for HIV latency utilize minimal genomes that lack various accessory proteins such as Nef, Vpr and Vif. Our previous work using lentiviral vectors that express Tat and Rev in cis but lack Nef has shown that epigenetic silencing of the proviral genome alters chromatin structures on the provirus and restricts initiation and elongation of proviral DNA. To evaluate the potential role of Nef gene in the establishment and maintenance of latency, we developed modified vectors that encode Nef under the control of an IRES sequence and inserted downstream of fluorescent reporter gene (LTR-Δgag-pol-Rev-Env-EGFP-IRES-Nef-LTR TatH13L). Jurkat cells infected with the lentivirus carrying Nef gene (Nef wt) are silenced faster than cells infected with viruses carrying a Nef knockout (Nef ko) or mutations in the myristoylation site of Nef (G2A) or tyrosine kinases binding domain of Nef (PXXP). Cells infected with viruses expressing Nef also showed a higher proportion of completely silenced cells that harbor latent proviruses. To evaluate the impact of Nef on proviral induction, we isolated representative clones of latently infected cells carrying the Nef wt, Nef ko and Nef mutants. As expected, the Nef wt cell clones, showed dramatically decreased MHC class I expression on cell surface following TNF-α stimulation. Interestingly, although both types of cell clones are able to be reactivated through stimulation of the T-cell receptor by anti-CD3/anti-CD28 stimulation, the Nef wt clones show enhanced expression of EGFP as monitored by flow cytometery analysis. We are currently performing a detailed analysis of the proviral reactivation in the presence of Nef using high resolution chromatin immunoprecipitation assays to monitor RNAP II and transcription factor recruitment to the proviral genome. In conclusion, we have demonstrated two distinct ways that Nef impacts HIV latency. First, following the initial infection, expression of Nef appears to enhance the rate at which cells are able to become latent, perhaps because of interference with cell signaling pathways due to the downregulation of surface receptors. Second, during the restimulation of latently infected cells Nef appears to enhance proviral transcription, perhaps by activating specific kinases.

THE REQUIREMENTS FOR NUP153 AND NUP358 DURING LENTIVIRAL INFECTION MAP TO THE CAPSID PROTEIN

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Lentiviruses are able to infect non-dividing cells, and thus likely utilize a mechanism whereby the preintegration complex (PIC) traverses the nuclear membrane through the nuclear pore complex (NPC). Although the viral and cellular factors involved in this process remain largely uncharacterized, recent results have suggested that the capsid (CA) protein, and thus perhaps viral core uncoating, are significant determinants of nuclear import. Furthermore, recent genome-wide RNA interference screens have highlighted numerous nuclear transport factors, including the karyopherin beta protein TNPO3 as well as a number of nucleoporins (NUPs) that comprise the NPC, as host-factors for HIV-1 infection. Follow-up results indicated that depletion of these proteins blocks HIV-1 infection at nuclear import or integration, and we moreover recently reported that the dependency on TNPO3 during infection maps to the HIV-1 CA protein.

To verify the importance of additional transport factors identified in the genome-wide screens, HeLa cells transfected with specific siRNAs were challenged with HIV-1 vectors, and resulting infectivities were normalized to those obtained in cells transfected with non-targeting siRNA controls. These results highlighted important roles for NUP153 and NUP358 in addition to TNPO3 during HIV-1 infection. Challenging the different knockdown cells with an expanded set of retroviral vectors indicated that primate lentiviruses including HIV-2 and several SIV strains were also significantly inhibited by TNPO3, NUP153, or NUP358 depletion whereas Moloney murine leukemia virus (MoMLV) and FIV were largely unaffected. By testing a panel of chimeric HIV-1 vectors encoding MoMLV matrix, CA, or integrase in place of their respective HIV-1 counterparts, we determined that substitution by MoMLV CA rendered the virus insensitive to NUP153 or NUP358 depletion. Furthermore, various HIV-1 CA missense mutants were differentially susceptible to NUP153 versus NUP358 knockdown; the cyclophilin A binding loop mutants G89V and P90A were insensitive to NUP358 knockdown, whereas the core-stabilizing mutant E45A appeared less sensitive to knockdown of NUP153 or TNPO3. Interestingly, NUP153 and TNPO3 depletion appeared to affect a similar set of CA mutant viruses, suggesting that these proteins may function in a common pathway during HIV-1 infection. Though an interaction between NUP153 and HIV-1 integrase was recently reported to be important for infection, our data instead highlight the CA protein as the dominant viral factor that determines the dependence on numerous nuclear transport factors including NUP153, NUP358, and TNPO3 during HIV-1 infection.

EVIDENCE THAT VPR USES THE DCAF1 UBIQUITIN LIGASE TO TARGET TWO HOST PROTEINS INVOLVED IN CELL GROWTH AND VIABILITY

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It is puzzling that so many viral auxiliary proteins exert pleiotropic activities despite their small size. Recent advances in the study of HIV-1 Vpu protein highlight a relevant mechanism whereby the viral protein bridges a host E3 ubiquitin ligase complex to distinct target proteins that are thus labeled for subsequent degradation. Similarly to Vpu, Vpr recruits a cullin-based ubiquitin ligase termed CRL4 through the DCAF1 adaptor subunit. This mechanism underlies the ability of Vpr to arrest cell cycle progression at the G2/M transition, leading to apoptotic cell death. Here we addressed whether Vpr-mediated effects other than G2 arrest might also rely on DCAF1 recruitment. We developed a clonogenic assay based on the expression of wild-type and mutant versions of Vpr using an EBV-based episome carrying the selectable marker hygromycin. Expression of the wt Vpr protein abrogated the formation of hygromycin-resistant cell colonies, confirming that long-term expression of Vpr is lethal in dividing cells. Intriguingly, two HIV-1 Vpr mutants, which are devoid of G2 arrest properties but conserve DCAF1 binding, strongly reduced the formation of hygromycin-resistant cell colonies. These mutants trigger apoptosis through caspase-3 activation but do not show any block in cell cycle progression. Thus Vpr expression can cause cell death independently of its G2 arrest activity as suggested by several studies. Disruption of the DCAF1-binding function of Vpr restored efficiency of colony formation indicating that recruitment of DCAF1 is required for this second cytotoxic activity of Vpr. However, DCAF1 binding is not sufficient to confer cytopathicity since Vpx from HIV-2/SIVsm, which also efficiently binds DCAF1, does not impair colony formation.
Altogether, our data indicate that Vpr exhibits two distinct cytotoxic activities in dividing cells which both require the recruitment of DCAF1. Based on the current view of its mechanism of action, Vpr likely induces the proteasome-mediated degradation of two host proteins independently required for proper cell growth and viability.

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O-GLYCOSYLATION OF PROTEINS ON A SURFACE OF T CELLS IS CRITICAL FOR HTLV-1 CELL-TO-CELL INFECTION

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We have previously described intron regulated retroviral reporter vectors that allow the quantitative measurement of cell-to cell transmission. We have shown that HTLV-1 infection is strongly dependent of producer-target cell contact, Tax expression, Env and cell type. Cell surface molecules involved in the formation of the virological synapse and viral transmission have been studied extensively by microscopy. However, their quantitative contribution to HTLV-1 replication remains poorly defined. In order to better characterize new or known cell surface antigens involved in cell-to-cell infection, we generated a panel of mouse monoclonal antibodies against Jurkat T cells and screened them in HTLV-1 and HIV-1 cell-to-cell infection assays. Among six inhibitory mAbs, three IgM class antibodies significantly decreased HTLV-1 cell-to-cell infection with no effects on HIV-1 infection. Immunoblot and mass-spectrometry analysis of proteins precipitated with these mAbs revealed that they recognize CD43 as well as CD45. Since both of these antigens are heavily O-glycosylated, we tested the mAbs in binding assays with synthetic saccharides and determined that they selectively bind Tn antigen. The Tn antigen is a monosaccharide N-acetylgalactosamine attached to serine or threonine residues in polypeptide chains. Normally this antigen determinant is masked by the elongation of the carbohydrate chain catalyzed by T-synthase, and as a result, most cells do not present Tn antigen. However, Jurkat cells bear a mutation in the gene C1GALT1C1 (COSMC), encoding T-synthase specific chaperon that is necessary for the proper folding of T-synthase. Consequently, O-glycosylation of proteins in Jurkat cells is interrupted. When we restored O-glycosylation in Jurkat cells by expression of wild type Cosmc, HTLV-1 cell-to-cell infection was enhanced 10 fold. Whether CD43 or/and CD45 antigens are critical for HTLV-1 infection will be determined in knock-down experiments. These findings imply a new role of heavily O-glycosylated proteins in cell-to-cell infection of HTLV-1 and are consistent with the recent data demonstrating that T lymphocytes transiently store HTLV-1 viral particles as carbohydrate rich extracellular assemblies.

SPROUTY2, A PI(4,5)P_2-INTERACTING PROTEIN, PLAYS A REGULATORY ROLE IN EVENTS REQUIRED FOR RELEASE OF HIV-1 GAG

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Sprouty2 (Spry2) is a PI(4,5)P_2-binding protein that cooperates with Alix but interferes with Tsg101 function in modulation of cell surface receptor down-regulation. Since PI(4,5)P_2 targets HIV-1 Gag to the plasma membrane, where Tsg101 or Alix facilitate Gag release, we determined whether Spry2 affected Gag trafficking. Here, we show that efficient release of the virus-like particles assembled from Gag required the steady-state level of endogenous Spry2. Moreover, in Cos-1 cells expressing Gag, adventitiously expressed Spry2 was translocated to the plasma membrane in a PTAP-dependent manner, implicating the Tsg101 bound to Gag in the recruitment. Gag and Spry2 co-localized at the periphery of some cells. However, under these conditions, much of the Gag was sequestered in the cell interior and release of the virus-like particles (VLPs) assembled by Gag was inhibited. The inhibition was relieved by mutation of the determinant of PI(4,5)P_2 binding in Spry2, suggesting that Gag and Spry2 compete for this phospholipid. Surprisingly, the inhibition of Gag release observed in the presence of Spry2 was relieved by disruption of the PTAP motif in Gag. The determinant of PI(4,5)P_2 binding in Spry2 and the YPX(n)L motif in Gag that recognizes Alix were required for this rescue. Co-immunoprecipitation, confocal microscopy and in vitro capture assays all indicated that Spry2 associates with ESCRT 2 factors and can interfere with their recruitment of ESCRT-1. The results identify Spry2 as a novel regulator of HIV-1 release that may facilitate Tsg101 and Alix interaction with ESCRT-3 by sequestering factors in the ESCRT-2 complex.

TARGETED LEDGF DOMINANT INTERFERENCE PROTEINS DEMONSTRATE CYTOPLASMIC EXPOSURE OF HIV-1 INTEGRASE

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HIV-1 infection is inhibited by target cell expression of the integrase binding domain (IBD) of the HIV-1 integration cofactor LEDGF. The structural requirements and mechanism for this dominant interference (DI) are unclear. More generally, it is not known whether integrase or other constituents within the Capsid-enclosed viral core become accessible to functional interaction with host cell proteins during cytoplasmic transit to the nucleus. Here we characterized parameters that determine DI protein activity and identified intracellular location effects with implications for the post-entry structural evolution and uncoating of HIV-1 particles. We determined that fusions of GFP to multiple IBD-containing segments of LEDGF inhibit HIV-1 replication, but a minimal GFP-IBD chimera, which distributes cell-wide, is most effective. Combining DI with endogenous LEDGF depletion using single step knockdown/re-expression lentiviral vectors was clearly multiplicative in effect, inhibiting single cycle infectivity by > 4 logs and completely blocking high (5.0) MOI viral replication in CD4 + T cell lines. We then sought to define when in the post-entry infection process integrase becomes accessible to GFP-IBD binding, by restricting the protein to specific intracellular locations. The protein retained potent antiviral activity when we confined it to either the nuclear envelope or to the cytoplasm. Moreover, the block triggered by a cytoplasm-confined DI protein manifests post-nuclear entry, at the level of integration. Certain other sub-cellular targetings caused loss of antiviral activity. These experiments demonstrate that LEDGF depletion and dominant interference target different temporal stages of the HIV-1 integration process and demonstrate that PIC integrase becomes accessible to cellular protein engagement in the cytoplasm.

ROLE OF HIV-1 Tat IN THE REGULATION OF VIRAL PREINTEGRATION TRANSCRIPTION

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HIV-1 preintegration transcription is the earliest viral transcriptional activity that generates three viral regulatory proteins, Tat, Nef, and Rev. Among them, Tat, the transactivator of transcription, is required for efficient viral transcription from the provirus, but its function in preintegration transcription remains largely unknown. To determine the involvement of Tat in preintegration transcription, we established an HIV-1 double mutant construct, pCMVΔ8.3(D116N/Δtat), a Tat expression vector, and an LTR-driven luciferase lentiviral vector. These constructs were used to assemble virion particles to characterize preintegration transcription. Using this system, we demonstrated that Tat is likely a part of the virion particle, and the virion-associated Tat is involved in the synthesis of non-integrated viral DNA. In addition, we also constructed a doxycycline-inducible Tat expression system. When introduced into HeLa cells, upon Tat induction, the Tat-mediated transcriptional activation predominantly acted on integrated proviral transcription, which exhibited a 12.5-fold increase in response to Tat; however, when non-integrated viral DNA template was identically tested, it displayed a significantly lower response, a merely 1.5-fold increase in reaction to Tat stimulation. Based on these results, we concluded that the virion-associated Tat is involved in viral DNA synthesis, but the early Tat protein synthesized from non-integrated viral DNA minimally stimulates preintegration transcription itself. The possible function of the early Tat protein is likely to stimulate viral transcription from subsequently integrated provirus.

EFFECT OF THE LENTIVIRAL ACCESSORY PROTEIN NEF IN HIV-1 PROTEASE ACTIVITY IN THE PRESENCE OF PROTEASE INHIBITORS

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Nef, an accessory protein expressed early during the replication cycle of the primate lentiviruses (HIV and SIV), plays an important role in viral infectivity and disease progression by a still not completely understood mechanism. Although many studies have described the role of Nef on the early stages of HIV-1 replication cycle, its contribution on the late stages is less explored. Our group has previously demonstrated that Nef interacts with GagPol by its p6*-PR region. Since both p6* and PR are involved with protein processing, we are now exploring Nef's role on maturation and viral protease activity. To assess the level of protease activity in the presence or absence of Nef, Hek293T cells were transfected with HIV-1 NL4-3 WT or deltaNef in the presence of Lopinavir (LPV), a HIV-1 protease inhibitor, at increasing concentrations or at a fixed concentration at different time points. Then, we observed the level of Gag processing in cell lysates and VLP's. We also assessed virus infectivity by the TZM-bl indicator cell line assay. In our experiments, NL4-3deltaNef was at least 10 times less sensitive to LPV than its WT counterpart, besides showing an abnormal processing profile and kinetics. The VLP's analyses also showed that NL4-3deltaNef has less unprocessed Gag. Our results indicate that in the absence of Nef the protease activity is increased, however, this event is deleterious to the virus, since NL4-3deltaNef is about 5 times less infectious than WT. Therefore, we suggest that premature activation of protease can lead to abnormalities, for instance in the ratio of proteins in the budding virus. This could indicate a regulatory role of Nef on HIV-1 Protease activity.

LIVE CELL VISUALIZATION OF INTERACTIONS BETWEEN HIV-1 GAG AND CELLULAR RNA-BINDING PROTEIN STAUFEN1

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As an obligate intracellular parasite, HIV-1 recruits host proteins and machineries to ensure abundant virus production and transmission to uninfected cells. Although host proteins involved in the transport of viral components towards the plasma membrane have been well investigated, the dynamics of this process remain incompletely understood. Previously we have shown that the dsRNA-binding protein, Staufen1 interacts with HIV-1 Gag and regulates its multimerization and assembly on membranes. In addition, Staufen1 is found in the context of a ribonucleoprotein (RNP) that contains the HIV-1 genomic RNA, Gag and several other host RNA-binding proteins such as NMD factor, Upf1, IMP1 and ABCE1. The formation of the HIV-1 RNP is dynamic and likely central to the fate of the genomic RNA during the late phase of the HIV-1 replication cycle.
In this work, we used bimolecular and trimolecular fluorescence complementation (BiFC and TriFC) to directly visualize the localization of protein-protein and protein-protein-RNA interactions in living cells. We show that wild type (wt)Gag and Staufen1 as well as wtGag and IMP1 interact in cells. These virus-host associations were not only present in the cytoplasm but were also found at GM-1-containing lipid raft domains at the plasma membrane. Importantly, in live and fixed cell assays, wtGag specifically recruited Staufen1 to the detergent insoluble membranes supporting a key function for this host factor during Gag assembly. In complementary TriFC experiments in which Gag was tethered to reporter mRNAs, strong interaction between both Gag-Staufen1 and the Gag-IMP1 was predominantly observed in the cytoplasm suggesting an active recruitment of host proteins when bound to mRNA.
The present work provides detailed information about where components of the HIV-1 RNP interact in cells and bring to light how HIV-1 recruits and co-opts RNA-binding proteins during the assembly of infectious particles.

HIV SUICIDE THROUGH VIRAL RNASE H

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We identified the RNase H of retroviruses and HIV as an essential enzyme for retroviral replication(1,2). It is specific for cleavage of RNA in RNA-DNA hybrids and required for removal of the viral RNA genome during DNA synthesis. We validated it as a target for drug design. Out of the four known retroviral enzymes it is the only one not yet targeted for by antiretroviral therapy. Reverse Transcriptase (RT) and RNase H resemble in structure and function PAZ and PIWI, components of RISC for antiviral defence by siRNA silencing (3). We are targeting the RNase H inside cell-free virus particles by the application of a short hairpin-looped oligodeoxynucleotide (ODN) driving HIV into suicide. A short local RNA-DNA hybrid forms as substrate for the RNase H which cleaves the viral RNA. This renders the particles non-infectious including HIV from patients and multi-drug-resistant strains (4-6). In a mouse with the oncogenic retrovirus, Spleen Focus Forming Virus, SFFV, intravenous treatment with ODN reduced the virus load, delayed disease progression or completely prevented infection (7). We also reduced the HIV load in the blood of 30 primary patients' isolates from Zurich and Africa by ex vivo treatment with ODN in more than 30% by 1000fold (8). Also the viral load in a mouse vagina can be significantly reduced in a prophylactic as well as therapeutic treatment (9). Humanized SCID mice are under study with HIV and ODN. Our approach is distinct from siRNAand antisense treatment in respect to mechanism, kinetics, and efficiency. It may prevent HIV infection during sexual or mother-to-child transmission or against multidrug-resistant viruses.
Also other viruses and cancer cells can be treated by activation of cellular RNases H.

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THE EFFICIENCY OF SEVI-MEDIATED ENHANCEMENT OF VIRUS INFECTION CORRELATES INVERSELY WITH THE NUMBER OF ENVELOPE GLYCOPROTEINS IN VIRAL PARTICLES

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We have shown that semen contains fragments of prostatic acid phosphatase that form amyloid aggregates termed SEVI (Semen derived Enhancer of Viral Infection), which interact with viral and cellular membranes and potently enhance HIV-1 infection by promoting viral attachment. Here, we further analyzed the mechanism underlying SEVI-mediated enhancement of virus infection. Predictably, SEVI had no effect on the infections rates of naked viruses. In strict contrast, SEVI drastically increased the infectiousness of HIV-1, HIV-2, various SIVs, FIV, KoRV, PERV, FeLV, MLV, XMRV as well as Marburg virus. Somewhat unexpectedly, however, SEVI only slightly enhanced infection by HSV-1, HSV-2, CMV, HBV, HCV, Dengue, RSV, VSV and Measles virus. Close examination revealed that all of the latter viruses contain a tightly packed glycoprotein shield that might render the viral membrane inaccessible for SEVI. In contrast, the membrane of retroviruses, such as HIV-1, contains only a low number of envelope (Env) glycoprotein spikes. To further assess the role of Env density on the virions for SEVI-mediated enhancement of viral infectivity we analyzed HIV pseudoparticles containing various amounts of heterologous VSV-G. We found a highly significant inverse correlation between the quantity of VSV-G on the virions and the magnitude of infectivity enhancement by SEVI. These results were confirmed using SIV particles carrying low, median and high amounts of SIV Env glycoproteins. Our results show that SEVI is a broad-based but not a general enhancer of enveloped viruses infection and suggest that the accessibility of the viral membrane is a prerequisite for effective SEVI-mediated infectivity enhancement.

EVOLUTIONARY PATHWAYS OF RALTEGRAVIR RESISTANCE ANALYZED BY DEEP SEQUENCING

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HIV integrase strand-transfer inhibitors like raltegravir are a new promising class of anti-retroviral compounds. As with all anti-HIV drugs, treatment with raltegravir sometimes leads to evolution of drug-resistance mutations (DRMs). Three pathways of raltegravir resistance have been reported, each with a different primary mutation. In this work, we investigated patients in whom limited clonal sequencing revealed that the viral population underwent pathway switches upon raltegravir administration. Deep sequencing with the 454/Roche pyro-sequencing technology was performed to gain more insight into the evolutionary dynamics of the DRM switch. We recovered 4058 sequences, from two patients analyzed longitudinally, corresponding to the HIV integrase coding region spanning most of the known integrase inhibitor DRMs. The sequences were condensed into operational taxonomic units (OTUs) following error-correction by *pyronoise*. In both patients, we confirmed the switch from the N155H to the more resistant Q148H + G140S pathway. In one patient this happened around 4 months while in the other this occurred around 12 months following treatment initiation. We found the primary mutations on multiple genetic backgrounds, indicating either multiple independent *de novo* generation or reassortment onto different backgrounds by recombination. Q148H-G140S eventually swept through the population in both subjects. In none of the patients were we able to detect any pre-existing polymorphism at codon positions 155 or 148 that could have been selected at later times. Finally even with high-resolution sequencing, we were unable to identify any double mutant N155H-Q148H variant. These findings emphasize the relatively greater fitness of the Q148H-G140S virus in the presence of raltegravir and document the evolutionary steps leading to the final resistant population.

INFLUENCE OF HUMAN APOBEC3F ON HIV-1 REPLICATION IN PRIMARY LYMPHOCYTES.

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APOBEC3 cytidine deaminases inhibit the replication of HIV-1 to different degrees in the absence of the viral protein Vif. For example, APOBEC3F (A3F) suppresses viral infectivity to a lesser degree than APOBEC3G (A3G) but it is more resistant to HIV-1 Vif-induced degradation. Multiple APOBEC3 molecules are expressed simultaneously in HIV-1 susceptible target cells but their individual contribution to viral suppression when expressed at endogenous levels remains largely unknown.
We used a replication competent HIV-1 NL4-3 molecular clone carrying Vif mutant W11R, which selectively counteracts A3G but not A3F, to dissect the relative contribution of A3F on HIV-1 replication inhibition in primary human lymphocytes. Contrary to our expectations, the NL4-3 W11R virus replicated to similar levels as NL4-3 WT in primary lymphocytes derived from five different healthy, HIV-1 negative blood donors. We sequenced A3F of these donors to assess for the presence of inactivating (partial or total) single nucleotide polymorphisms. We found three different A3F haplotypes, one of which showed substantially lower antiviral activity when compared to the two others in single cycle infection experiments. However, when we quantified the A3F mRNA expression in the same PBMC samples by qPCR, we observed low expression levels in all 5 donors.
Taken together our data indicate that despite differences in antiviral activity among A3F haplotypes, the endogenous A3F expression in primary lymphocytes is limited to the extent that its neutralization is dispensable for spreading infection of HIV-1.

A ROLE FOR RAC1 AND RHOA GTPASES IN HIV-1 GAG ASSEMBLY AND RELEASE.

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Our aim was to characterize the RhoGTPases and cell signaling pathways, which are specifically modulated by the process of HIV-1 assembly and production. Along this line, it has been proposed that these steps involve membrane and actin cytoskeleton remodeling which is regulated by the small RhoGTPases. In fact, a number of data suggests the implication of such proteins in HIV-1 production. Indeed, the use of toxin B, a drug which suppresses Rho GTPase activity, decreases HIV-1 release. In addition, HIV-1 virions contain some RhoGTPases and actin-binding proteins.
Our preliminary results reveal that Rac1 and RhoA GTPase proteins can impact on HIV-1 Gag membrane attachment and localization, and viral production. Thus, Gag assembly and viral production might require a transitional regulation of cell signalling pathways mediated by RhoGTPases. However, the molecular mechanism underlying this effect is not yet understood. What is the role of the RhoGTPases in HIV-1 assembly and budding?
Gag assembly takes place at the level of membrane microdomains such as the tetraspanin CD81-enriched microdomains at the plasma membrane, the raft or the PI(4,5)P2 microdomains where HIV-1 virions are released by budding. In addition, these microdomains are the sites where activated RhoGTPases can be anchored, and which determine cell endocytosis and migration, and membrane-cytoskeleton regulation via actin-mediated cell signaling. During Gag interaction with these membrane microdomains, the Gag proteins could well recruit and modulate the activity of RhoGTPases, such as the main Rac1, Cdc42 and/or RhoA GTPases, in order to facilitate virus assembly and release.
We, thus, studied the implication of these RhoGTPases in virus assembly and release. In HIV-1 producing cells, the RhoGTPases of interest were either suppressed or overexpressed and for each conditions, we analysed the intracellular Gag localization, viral assembly and maturation, and viral production by several methods (RT test, immunoblots, membrane flotation assays) and by cellular imaging (confocal microscopy). The overexpression of Rac1 or RhoA Dominant Negative mutant proteins reduces viral production, modifies intracellular Gag localization and reduces Gag membrane attachment. In addition, the overexpression of Rac1 or RhoA WT or Dominant Positive mutant proteins enhances viral production while inhibition of endogenous Rac1 and RhoA by siRNA or by specific drugs impacts viral production.
Our results show that regulation of cell signaling by RhoGTPases is required for HIV-1 assembly and release. We will thus determine which are the cellular RhoGTPase mediated pathways and their interactions with viral components involved in HIV-1 assembly and release.

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DUPLICATION OF THE BST2/TETHERIN GENE IN THE SHEEP GENOME.

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The colonization of the sheep genome by JSRV-related endogenous retroviruses (enJSRVs) is a process that is still ongoing at the present time. Several of the recent enJSRV proviruses are insertionally polymorphic, maintain an intact genomic structural organization and are able to release viral particles in vitro. enJSRVs are abundantly expressed in the uterus during pregnancy, proving essential for conceptus development and placental morphogenesis. Interferon tau (IFNT), a type I interferon, is the pregnancy recognition signal secreted by the ovine developing conceptus prior to implantation. Previously, we have shown that bone marrow stromal cell antigen 2, (BST2/tetherin) is upregulated by IFNT in sheep cells (Arnaud et al, JVI 2010, Feb 24). We have also found that enJSRVs and oBST2 expression peak simultaneously during pregnancy but they are expressed in different cellular compartments of the sheep uterus. By transient transfections we showed that oBST2 inhibits viral particle release of enJSRVs and we proposed that oBST2 may have been an important factor in shaping the co-evolution of enJSRVs with their host. Interestingly, we found that oBST2 is duplicated both in the sheep and cattle genome. oBST2-A appears to have a stronger antiviral activity than oBST2-B. oBST2-A and oBST2B display a different intracellular distribution as assessed by immunofluoresence. In addition, the enJSRVs Env displays a different intracellular distribution whether expressed in the presence of oBST2A or oBST2B. The biological significance of the differences observed in oBST2A and oBST2B and the possibility of ruminant-specific antiviral mechanisms are currently being investigated.

ROLE OF ZINC FINGERS OF HIV-1 GAG IN NUCLEIC ACID BINDING AND CHAPERONE FUNCTION

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The HIV-1 nucleocapsid (NC) domain of Gag has both specific and more general nucleic acid (NA) binding properties. NC recognizes the specific Ψ packaging signal on the genome, thereby ensuring that unspliced viral RNA is packaged into virus particles. In addition, NC acts as a general NA chaperone to facilitate reverse transcription by annealing/aggregating NAs via its highly positive character, and by destabilizing NA secondary structure via its two CCHC zinc finger (ZF) motifs. Interestingly, it has recently been reported that ZF deletion or mutation to CCCC results in production of virions containing DNA instead of RNA, rendering them noninfectious (1, 2). Thus, an additional role of NC is to prevent premature reverse transcription from occurring before or during assembly. Here, we probe the in vitro NA binding and chaperone properties of Gag variants containing the same ZF mutations or deletions tested in the cell-based assays. Fluorescence anisotropy equilibrium binding measurements reveal that mutation or deletion of both ZFs results in a modest reduction (~ 3-fold) in binding to the Ψ SL3 stem-loop relative to WT Gag, whereas binding to nonspecific single-stranded NAs is largely unaffected. Similarly, Gag's ability to aggregate NAs or to facilitate tRNALys3 annealing to the primer-binding site, two functions of Gag during viral assembly, was only moderately affected (~ 3-fold) upon ZF mutation or deletion. A time-resolved fluorescence resonance energy transfer assay was used to monitor hairpin stem opening of a doubly dye-labeled TAR DNA construct. Surprisingly, single CCCC and ZF deletion variants were more effective at opening the TAR hairpin than WT Gag, and Gag variants in which both zinc fingers were mutated or deleted showed even greater duplex destabilization capability than the single ZF variants. Previous studies with the freestanding NC domain of Gag indicated that NC's duplex destabilization activity depended on the ZF structures. Our new results suggest that in the context of Gag, disruption of the ZFs leads to enhanced duplex destabilization and additional studies to confirm these findings are underway.

1. Thomas et al., J. Virol. 82: 9318-9328, 2008
2. Houzet et al., Nucleic Acids Res. 36: 2311-19, 2008

VRNA AND PI(4,5)P2 DRIVE POLYHEXAMERIC OLIGOMERIZATION OF RSV GAG

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Rous sarcoma virus (RSV) assembly depends on a series of spatio-temporally coordinated steps that culminate in the budding of spherical particles from the plasma membrane (PM). Assembly begins with Gag:viral RNA (vRNA) binding, followed by multimerization of Gag into a hexameric lattice at the PM. Here, we report that Gag binding to vRNA in vitro promotes poly-hexameric complex formation; however, Gag:vRNA interaction is not sufficient to trigger spherical particle formation at neutral pH. To determine whether an additional cellular factor was required, we investigated the role of PM-localized phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2], previously implicated in targeting of the HIV-1 and MuLV Gag proteins.

To test whether PI(4,5)P2 is required for RSV Gag PM localization and budding, 5-phosphataseIV (5ptaseIV) was expressed to deplete intracellular levels of PI(4,5)P2. In cells that co-express the RSV genome and 5ptaseIV, Gag accumulated in cytoplasmic foci and virus particle production was decreased. In vitro binding experiments revealed that Gag and the isolated MA protein bound directly to PI(4,5)P2 with high affinity (Kd=74nM and 147nM respectively). Additionally, PI(4,5)P2 binding induced a conformational change in both MA and Gag. Surprisingly, PI(4,5)P2 changed the oligomeric state of MA from monomer to hexamer, suggesting that MA-membrane interactions promote formation of the hexameric lattice. In contrast, Gag:PI(4,5)P2 remained monomeric unless vRNA was added, resulting in polymerization into tube-like structures apparently composed of hexameric rings.

In conclusion, we show that targeting of RSV Gag to the PM is mediated by PI(4,5)P2 and vRNA interactions, in contrast to myristylated HIV Gag, which requires structural rearrangement of the myristate moiety and PI(4,5)P2 binding. Furthermore, PI(4,5)P2 and vRNA binding have a synergistic effect on Gag polymerization in vitro, driving the formation of an apparent hexameric lattice. We propose that upon binding to vRNA, Gag forms hexameric complexes that migrate to the PM where tight binding to PI(4,5)P2 occurs. Gag:vRNA:PI(4,5)P2 binding induces conformational changes that might recruit other Gag molecules to form complete virus particles. Gag:vRNA:PI(4,5)P2 forms tubes rather than spheres, possibly due to the lack of an additional host factor or a curved membrane surface. Future experiments will test these possibilities, leading to a deeper understanding of the biochemical interactions that drive retrovirus particle assembly.

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PROTEASOME-DEPENDENT AND –INDEPENDENT RESTRICTION OF HIV-1 BY TRIM5A

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Background: TRIM5α contains RING domain, which is frequently found in E3 ubiquitin ligases. It was reported that mutations disrupting RING domain function of rhesus monkey (Rh) TRIM5α only partially diminished its anti-HIV-1 activity. Although it has been proposed that Rh TRIM5α restricts HIV-1 via proteasome-dependent and -independent pathways, the precise role of RING domain in retroviral restriction remains unclear.
Methods: We constructed mutant TRIM5αs in which Zn-binding sites of RING domain were disrupted and examined the effects of these mutations on retrovirus restriction by TRIM5α in various combinations of virus and host species.
Results: SIVmac successfully avoided attacks by RING mutants of African green monkey (AGM) TRIM5α that could still somewhat restrict HIV-1 infection. Addition of proteasome inhibitor MG132 did not affect the anti-HIV-1 activity of AGM TRIM5α at all, whereas it disrupted at least partly the anti-SIVmac activity. These results indicated that AGM TRIM5α restricted SIVmac mainly via the proteasome dependent pathway, whereas HIV-1 restriction by AGM TRIM5α was both proteasome dependent and independent. In the case of human TRIM5α carrying proline at the position 332 (Hu-R332P), however, both HIV-1 and SIVmac restrictions were completely eliminated by RING domain mutations. Furthermore, a proteasome inhibitor could also disrupt both anti-HIV-1 and anti-SIVmac activities of Hu-R332P. These findings indicate that Hu-R332P restricts both HIV-1 and SIVmac mainly via the proteasome dependent pathway. When we measured levels of reverse transcribed (RT) products of HIV-1, both late RT products and 2 LTR forms were severely suppressed in cells expressing parental TRIM5αs while those in cells expressing RING mutations of AGM and Hu-R332P TRIM5αs were significantly recovered.
Conclusion: The mechanisms of retrovirus restriction by TRIM5α vary depending on the combination of host and virus species.

FORCED STOCHASTIC BEHAVIOR OF VIRAL COMPETITION EXPERIMENTS PROVIDES METHOD TO INFER EFFECTIVE POPULATION SIZE.

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Treatment of HIV infection *in vivo* is complicated by the rapid evolution of drug resistance mutations. Attempts to model the evolution of HIV fall into two general categories, deterministic for large populations and stochastic for small populations. Importantly, it is not the census population size but the effective population size (N_e) that is the relevant parameter in these models. N_e is difficult to directly measure because it is influenced by bottlenecks, metapopulation structure, variance in progeny number, etc., but can be inferred by calibrating the predictions of a reference model, in which N_e is a parameter, to observable behavior in an experimental system.

We developed a tissue culture based system using ALV that closely meets the assumptions a simple model to describe the evolution of a population containing two variants, which differ in fitness due to a single point mutation in *env* with a choice of two alleles. A defined mixture of the two viruses is passed repeatedly in culture, and their relative frequency as a function of passage number is assessed by allele-specific PCR. By making the better-fit virus increasingly rare at the start of the competition we are able to observe the evolution of the population shift from deterministic to stochastic evolution, as indicated by the increasing variance of the rate of fixation of the more fit variant, a behavior described by the model. We found that the magnitude of stochastic behavior is a function of the selection coefficient between the two competing viruses, another prediction made by the model.

We propose that the ratio of N_e to N (i.e., the effective to the census population size) that describes this simple experimental system will provide an upper bound to inferences of N_e when this model is applied to the more the complicated system of HIV infection *in vivo*.

GENOMIC RNA STRUCTURES MINIMISE RECOMBINATION COST THROUGH GENE SWAPPING IN HIV

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HIV uses RNA structures in its genome, including the RRE, TAR and dimerization sequences, to modulate and regulate its replication cycle. The structure of a full length genomic RNA of the NL4-3 molecular clone of HIV was recently described (Watts et al. Nature 2009). Interestingly, the relatively unstructured protein sequences linking autonomously folding protein domains were found to be encoded by highly structured regions in the RNA genome. This distribution of RNA structure suggested a model in which these structures facilitate native folding of HIV proteins.
As, in HIV, RNA structures have also been associated with the promotion of recombination in mechanistic studies, creating local recombination hot spots, we compared the distribution of RNA structure to the variations in recombination rates in the envelope gene. We observe that the recombination pattern in env, obtained using primary isolates of different HIV-1 subtypes in a system where recombination is analyzed in the absence of selection (Simon-Loriere et al, PLoS Path. 2009), is clearly recaptured by the presence of RNA structures. The pattern of RNA structure is thus a strong predictor of genome regions with high levels of recombination in HIV-1.
If this observation, made in env in absence of selection, can be extended to the rest of the genome, then traces of the distribution of RNA structure should be observed in the pattern of recombinant breakpoints observed in nature. This is particularly true in regions of the genome where recombination presents a lower risk of disrupting intra-gene interaction networks such as in regions between genes or proteins, or between protein domains.
We do observe significantly higher levels of breakpoints in structured regions of the RNA genome and in intergenic regions; breakpoints in the former category clearly dominating on the second. In parallel, we also observe a noticeable trend for an enriched presence of structured RNA regions to be located preferentially in intergenic regions. Recombination is thus oriented such that the probability that recombination results in functional proteins is maximized. As a result, recombination between divergent sequences, such as between genomes belonging to different HIV-1 subtypes, tends to result in the transfer of entire genes to a different genetic background, rather than creating chimeric genes.

TETHERIN UBIQUITINATION AND DEGRADATION ARE NOT ESSENTIAL FOR VPU-MEDIATED ANTAGONISM OF ITS ANTIVIRAL ACTIVITY

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Antagonism of tetherin/CD317-mediated restriction of retroviral particle release by the HIV-1 accessory protein Vpu results in the downregulation of tetherin levels from the cell surface and its degradation. Vpu-induced tetherin degradation, but not downregulation of cell surface levels, can be rescued by proteasome inhibition indicating it is an ubiquitin (Ub)-driven process, although whether its ultimate destruction occurs in the lysozome or after dislocation to the cytoplasm remains unclear. The cytoplasmic tail of human tetherin contains two membrane proximal lysine residues (K18 and K21) that could serve as potential Ub-acceptors. Indeed K18 is essential for the targeting of tetherin for ESCRT-dependent degradation by K5, a RING-CH E3 ligase encoded by Kaposi's sarcoma-associated herpesvirus (KSHV). Ubiquitinated species of tetherin can be detected in Vpu-expressing cells, and this is dependent on the presence of K18 and K21. Using HT1080 cells stably co-expressing tetherin mutants and Vpu, we can show that degradation of tetherin by Vpu depends primarily on K21, although K18 mutants display partial resistance to degradation. K21R mutants of tetherin were also poorly downregulated from the cell surface. Despite these defects in degradation and downregulation of tetherin K-mutants, there was little or no difference in the abilities of these proteins to restrict HIV-1 particle release, or their sensitivity to Vpu-mediated antagonism. Thus Vpu-induced ubiquitination and degradation of tetherin is dispensable for blocking its antiviral activity.

INVOLVEMENT OF THE ENDOGENOUS HERV-F(C)1 RETROVIRAL LOCUS ON THE HUMAN X CHROMOSOME IN THE ETIOLOGY OF MULTIPLE SCLEROSIS

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Two views have dominated the discussions on the etiology of multiple sclerosis in recent decades: It could be a genetic disease, or it could be a disease caused by an infectious agent. The genetic view is most clearly expounded by studies of twins, showing that concordance among monozygotic twins is approximately 25 percent, while concordance among dizygotic twins is only 2 - 3 percent. The alternative view, that multiple sclerosis is caused by an infectious agent, is most clearly backed by animal studies. The obvious intersections of a genetic and a viral etiology are the human endogenous retroviruses.

48 intact or near-intact human endogenous viruses were treated as Mendelian loci in an analysis based on genetic epidemiology. We investigated DNA from 350 patients with verified multiple sclerosis living as well as DNA from 500 persons without sclerosis from the same part of the country. Females constituted 62 percent of cases and 67 percent of the controls. A total of 220 markers were tested. A particularly striking cluster of significant markers occurred on chromosome X at approximate chromosome position 97100000 around the HERV-F(c)1 proviral locus (NCBI genome build 37.1). The marker rs391745 was lowest with a p-value of 4*10-6 (2-sided) for association with disease, when calculating on the basis of C-allele carriers. This value remained significant after Bonferroni correction (p = 0.0009).

We retested the association of rs391745 and multiple sclerosis in another cohort of 542 verified cases and 1160 controls, making sure that there were no overlaps between the two cohorts. Females constituted 66 percent of cases and 65 percent of the controls. Rs391745 was again associated with MS. Finally, we tested a third smaller cohort of verified multiple sclerosis in Eastern Danes. In this cohort women constituted 70 and 63 percent among cases and controls, respectively. We could not find any association of rs391745 with multiple sclerosis, and do not know why the association was absent. The p-value for all 3 cohorts combined was 0.00001 (2-sided) and thus significant after Bonferroni correction (p = 0.003).

We performed a scan of the region surrounding the HERV-F(c)1. It was clear that the association occurred in a 20 kb region around the provirus. In contrast, the nearest known genes lie 141 kb upstream, and 57 kb downstream, respectively.

By treating the endogenous retroviruses as Mendelian loci, we have brought the formidable knowledge of human genetics to bear on their role in disease, and avoided the difficulties of establishing the causality of a ubiquitous agent.

ELEVATED STRAND TRANSFER BY MULTIPLE NRTI RESISTANT HIV-1 RT MUTANT HARBORING A TWO AMINO ACID INSERTION IN THE FINGERS DOMAIN.

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HIV-1 recombination events are a major contributor to creating viral diversity. Recombination happens approximately 20% of the time between the two single stranded RNA genomes of HIV-1 during reverse transcription (Onafuwa-Nuga et al. 2008). We are searching for viral mutations that affect the strand transfer activity of HIV-1 RT known to mechanistically contribute to viral recombination. For this, two RT clinical isolates, a pre-drug RT isolate and post-drug RT isolate from a single patient before and after multiple NRTI treatments, respectively, were used to study strand transfer efficiency with different RNA substrates during reverse transcription. The RNase H activities and RNA template degradation of these two RT isolates during reverse transcription were also assayed to coincide with strand transfer activity observed in both RT isolates. We found that the post drug RT isolate containing multiple thymidine analog mutations (TAMs) along with a dipeptide finger insertion mutation SG at positions 69 and 70 have increase strand transfer efficiency during reverse transcription compared to the pre-drug RT isolate. There were also more primary and secondary cuts generated from the post drug RT isolate in the RNase H assay. We hypothesize that the post drug RT isolate having the finger dipeptide insertion along with the background TAMs alters its interaction with the template, and possibly affects strand transfer of HIV-1 RT. This enhancement in strand transfer frequency of HIV-1 may allow the virus to effectively combine various existing mutations in a round of replication, ultimately promoting the evolution and escape of HIV-1 from multiple anti-viral selective pressures.

A NOVEL HOST FACTOR, UBIQUITIN-CONJUGATING ENZYME E2 VARIANT 1 (UBE2V1), INFLUENCES HIV-1 INFECTION IN HUMAN MONOCYTIC CELLS

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Host factors play a critical role in HIV-1 replication and cellular restriction of viral infection. Studying the mechanisms of HIV-1 interaction with host factors can facilitate our understanding of viral pathogenesis. The cell-type specific signaling pathways and the associated transcription factors can influence the infection by HIV-1. In our effort to explore postentry restriction of HIV-1 infection in undifferentiated human monocytes, we identified a novel host factor, ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1), which significantly influences HIV infection in certain cell types. UBE2V1 belongs to the subfamily of ubiquitin-conjugating E2 enzyme variant proteins, which are catalytically inactive forms of E2 enzymes. The role of UBE2V1 in HIV-1 infection has been analyzed using a human promonocytic cell line, U937. Compared to parental U937 cells, a stable small hairpin RNA (shRNA) based knock-down of UBE2V1 in U937 cells resulted in a 10±1 fold increase in HIV-1 gene expression upon infection with a single-cycle, vesicular stomatitis virus G protein (VSV-G) pseudotyped NL4-3 luciferase reporter virus. Moreover, infection with a replication-competent NL4-3 Δnef-luciferase HIV-1 resulted in greater than a 40-fold increase in the luciferase gene expression. The fold increase in HIV-1 infection levels is observed to be independent of the multiplicity of infection. In contrast, shRNA-based stable knock-down of UBE2V1 in human CD4+ T cell line Sup-T1 did not significantly affect the infection with the replication-competent virus. There is a slight decrease (20±5%) in luciferase expression in the Sup-T1 cells infected with the single-cycle HIV-1. The role of UBE2V1 in HIV-1 infection of primary monocytes, macrophages, and CD4+ T cells is under investigation. A UBE2V1/UBE2N heterodimer is known to have a role in the signaling by TNF receptor associated factor 6 (TRAF-6), transcriptional activation of target genes, and cell cycle and differentiation. Details of the events in HIV-1 life-cycle that are influenced by UBE2V1 will be presented. Our results suggest that UBE2V1 may act as a cellular regulator for HIV-1 gene expression in certain cell types.

BARRIERS TO INTERGROUP HIV-1 RECOMBINATION REVEALED BY A NEW DUAL-MARKER SYSTEM

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High rates of HIV-1 recombination generate diversity in circulating strains and allow the virus to escape immune responses and develop drug resistance. We previously showed that the dimerization initiation signal (DIS) identity affects the copackaging efficiency of RNA derived from different proviruses. For example, HIV-1 subtypes B and C have different DIS sequences and their RNAs are copackaged less efficiently than those with the same DIS. These conclusions came from studies using the reconstitution of a functional green fluorescent protein gene (*gfp*) during recombination as a surrogate. We have now established a dual-marker system that uses the reconstitution of a functional Gag (capsid) protein, in addition to *gfp*, to directly detect recombination in the viral genome.

Using this system, we have shown that recombination occurred more frequently in the *gag* gene between two viruses of the same subtype (B/B and C/C) than between viruses of different subtypes (B/C), even when the viruses contained the same DIS. These results revealed that aside from DIS identity, there were other barriers to HIV-1 intersubtype recombination.

In addition to group M viruses, which includes both subtype B and C variants, there are three other HIV-1 groups, O, N, and P. Despite the high genetic diversity, group M and group O recombinants have been previously reported in clinical samples. We are currently studying the barriers to intergroup HIV-1 recombination. Our preliminary results suggest that when containing the same DIS, group O RNA copackages less frequently with group M RNA compared to RNAs from two different viral subtypes. Additionally, recombination in the *gag* gene occurs less frequently between group O and group M viruses than between two subtype B or two subtype C viruses. Therefore, multiple barriers exist in the generation of intergroup HIV-1 recombination.

* Equal contribution

RECONSTRUCTION OF HERV-F(C)1, ASSOCIATED WITH MULTIPLE SCLEROSIS

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The complex and still obscure etiology of Multiple Sclerosis (MS) seems to involve hereditary and environmental, probably infectious, factors alike. Human endogenous retroviruses (HERVs), inherited as mendelian genes, could be the link between these factors.
A recent association study in our group investigated association between Multiple Sclerosis and all HERVs with potential for (or close to) protein expression. This analysis revealed association in a region of chromosome X containing HERV-F(c)1.
The HERV-F(c)1 sequence contains the general retrovirus structure of LTR-elements and the three genes Gag, Pol and Env. The Env gene seems intact with an open reading frame, whereas the Gag and Pol frames are interrupted by two premature stop-codons and a frameshift mutation. Thus, we suspect that only three point mutations keep this provirus remnant from having expression potential for all three viral proteins. In order to gain more knowledge about the capacity of the virus and particles derived from it, we decided to reconstruct the potential for Gag-Pol polyprotein expression of HERV-F(c)1 by means of site-directed mutagenesis.
BLAST-search comparison with other viral sequences indicated the most probable nucleotides for substitution (point-mutation) or deletion (frameshift). The sequences for HERV-F(c)1 Gag-Pol and Env were cloned into two different expression vectors respectively. This minimizes the risk of replication competent retrovirus (RPR) production upon concurrent expression and virion production. The expression vectors allow for various future studies revealing the nature
of this virus upon reactivation.

LENS EPITHELIUM DERIVED GROWTH FACTOR (LEDGF) FUSION PROTEINS THAT REDIRECT HIV-1 DNA INTEGRATION

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We and others have shown that fusion proteins in which the PWWP domain and AT hooks of LEDGF are replaced by other chromatin binding domains redirect HIV-1 integration. There are two reasons to continue to pursue this technology 1) to control the specificity of integration for safer gene therapy 2) to understand the binding specificity and distribution of chromatin binding proteins. We have prepared several new LEDGF fusions that alter the integration pattern of HIV DNA in KO mouse cells that lack normal LEDGF. We previously showed that a PHD finger from ING2 (which binds H3K4Me3) fused to the integrase binding domain of LEDGF preferentially directs integrations near the transcriptional start sites (TSSs) of expressed genes. We now show that a PHD finger from JARID1α (which also binds H3K4Me3), causes a similar distribution and, with some interesting exceptions, favors integrations in the same genes. In contrast, a fusion with a domain from MLL containing both a PHD finger that binds H3K4Me3 and an atypical bromodomain, directs integrations near the TSS of a very different group of expressed genes than the ING2 and JARID1α PHD finger fusions. We reported the distribution of integrations directed by the chromodomain of HP1α. We now show that a fusion with the chromodomain from Cbx7, which recognizes a related set of histone modifications, directs a pattern of integration that is distinct from the HP1α fusion. These new data extend our previous work and show that a combination of domains with different chromatin binding specificity can lead to novel patterns of integration, a result that holds promise both for gene therapy and for the study of the specificity of chromatin binding proteins.

RECOMBINANT TY3 INTEGRASE MEDIATES BRF1C-DEPENDENT AND SEQUENCE-SPECIFIC STRAND TRANSFER IN VITRO

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Ty3 is a long terminal repeat retrotransposon in the budding yeast *Saccharomyces cerevisiae*. Ty3 integration occurs preferentially at the transcription initiation sites of genes transcribed by RNA polymerase III (Pol III). Two general transcription factors, TFIIIB and TFIIIC are required by Pol III *in vivo*; however, *in vitro* at the *SNR6* gene, which contains an upstream TATA element, TFIIIB is sufficient to support transcription on a naked DNA template. Previously, using Ty3 VLPs as the source of integrase (IN) and cDNA, our laboratory showed that Ty3 integrated at low but detectable frequencies in the presence of TFIIIB subunits Brf1 and TATA-binding protein (TBP). In this study, we purified recombinant Ty3 IN and characterized its catalytic activity. A Brf1n-TBPcore-Brf1c triple fusion protein (TFP) was shown to be able to replace Brf1 and TBP for TFIIIC-independent transcription [1]. Using the TFP construct, a novel integration assay was developed in which recombinant IN mediated strand transfer of oligonucleotide duplexes representing the Ty3 termini to sites of TFP binding. Electrophoretic mobility shift assay (EMSA) showed the formation of a complex consisting of IN, TFP, and a TATA box-containing DNA in the presence of Mg^{2+}. GST pull-down assays were used to show that the interaction was between the Brf1c domain of TFP and the C-terminal domain of IN. When Mn^{2+}, an alternative cofactor for retrovirus IN, was supplied in the reaction, TFP targeting was abolished but new insertion sites were found to concentrate in an upstream region. Examination of nearby sequences in that region revealed an 8-bp stretch almost identical to Ty3 LTR end sequence. Based on this result, we hypothesize that in the absence of targeting factors, Ty3 IN may bind to target sequences resembling its own termini and have weak strand-transfer activity.

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MOLONEY MURINE LEUKEMIA VIRUS INTEGRASE (M-MULV IN): STRUCTURAL STUDIES OF THE N-TERMINAL DOMAIN

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The M-MuLV IN protein is a 45kDa protein, and thus encodes insertions beyond the three conserved domains: HHCC zinc binding domain at the N-terminus, the catalytic core, and the C-terminal domain. Of particular interest was the MuLV IN N-terminal domain (NTD: amino acids 1-105). Using NMR, X-ray crystallography, and SAXS analysis, we find that in addition to the structurally conserved HHCC zinc binding region, the NTD contains an N-terminal extension domain (NED) similar to PFV IN. While the NED is not conserved among many retroviruses, this region is essential for M-MuLV integration, both in vitro and in vivo. In solution, the M-MuLV IN NTD forms a dimer with a Kd of ~650 nM. A selective labeling strategy, including the production of asymmetrically labeled dimers, has led to the elucidation of residues involved in the dimer interface. While the X-ray and NMR structure display concordance within the monomer, a similar dimer is not present in the X-ray crystal structure. To further characterize the dimer SAXS analysis was performed. Mutational analysis, based on the structure of the NTD, has highlighted critical amino acids required in vivo for viral infection, where were previously found non-essential for in vitro integration assays. Comparison of these structures, as well as with the structure of the NED/NTD of the PFV IN in complex with DNA will be presented.

LEDGF/P75 IS CRITICAL BUT NOT ESSENTIAL FOR MULTIPLE ROUND REPLICATION OF HIV-1 IN HUMAN KNOCKOUT CELLS.

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During HIV-1 replication the cellular cofactor LEDGF/p75 (Lens Epithelium Derived Cofactor/p75) binds HIV-1 integrase and targets viral integration preferentially into active genes. Several strategies were used to show the important role of LEDGF/p75 in viral replication. After RNAi-mediated knockdown of LEDGF/p75, residual replication was observed possibly supported by minute LEDGF/p75 protein levels. Mouse knockout fibroblasts were generated enabling analysis of high titer single round lentiviral vector transduction but not of multiple round replication. To enable evaluation of multiple round replication in the complete absence of LEDGF/p75, a human LEDGF/p75 somatic knockout cell line was generated (-/-) leaving the p52 splice variant intact.
By homologous recombination in the pre-B-cell line Nalm-6, exons 10 to 13, coding for the LEDGF/p75 integrase binding domain (IBD) were deleted. As a result a truncated protein was formed in which the C-terminal region of LEDGF/p75 (aa 325-530) was replaced by a new 9 aa tail. Correct homologous recombination was verified by southern blot analysis and DNA sequencing. Absence of LEDGF/p75 specific mRNA was verified by Q-PCR. Western blot analysis revealed the presence of the truncated protein. Multiple round HIV replication in LEDGF/p75 -/- and +/- cells was delayed for 2 weeks in the LEDGF/p75 null cells. Using 454 sequencing the HIV and EIAV integration site profile was determined. In LEDGF/p75 +/+ cells 74 % of the HIV integration sites were found in Refseq genes compared to 48 % in the -/- cells. For EIAV integration, these numbers were 60 % and 37 %, respectively. In addition, upon knockout of LEDGF/p75, HIV-1 and EIAV integration around CpG islands (1 kb) increased 6- and 4-fold, respectively. These results are in line with earlier reports investigating integration site preference in human LEDGF/p75 knockdown cells or mouse knockout cells.
Our results corroborate LEDGF/p75 as a critical, but not essential cofactor for HIV replication in human cells. We are currently evaluating LEDGF/p75 knockout escape mutants to understand how HIV is capable to replicate in human cells in the complete absence of LEDGF/p75.

HOST FACTORS IN HIV INTEGRATION SITE TARGETING

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Recent siRNA screens have identified numerous human genes whose expression is necessary for efficient HIV infection. A number of identified factors are required for nuclear import and/or integration of the virus, and it has been suggested that these steps may be mechanistically coupled. We have investigated the role of several nuclear factors in HIV integration using siRNA to deplete protein levels and 454-pyrosequencing of 13,329 unique integration sites to map proviral distribution. Genomic sequences flanking proviruses were aligned to the hg18 draft human genome sequence and compared to annotated features including genes, exons, GC content, and CpG islands. Depletion of TNPO3, RANBP2 (NUP358), ANAPC2, and SNW1 significantly reduced HIV integration targeting to gene-rich and G/C rich regions, compared to wild-type HIV integration sites. This pattern was distinct from that seen when PSIP1/LEDGF/p75 is depleted. As a control, we investigated integration targeting of MuLV, which is not thought to traverse the nuclear pore, and whose integration steps are LEDGF-independent. The distribution of MuLV proviruses was not altered with respect to gene or G/C density when TNPO3, ANAPC2, and SNW1 were depleted. In agreement with previous reports, depletion of these three factors inhibited HIV infection while only the latter two reproducibly prevented MuLV infection. Effects of knockdown on infectivity and integration targeting were partially rescued by expression of siRNA-insensitive alleles of TNPO3 and ANAPC2. Given that TNPO3 and RANBP2 are components of nuclear import pathways, their necessity for normal integration targeting of HIV but not MuLV is consistent with a model of linked import and integration. Our data is in agreement with concurrent studies with collaborators showing that capsid mutants defective in binding RANBP2 also have altered integration site selection. How these factors interact with the PSIP1/LEDGF/p75 pathway, which also affects targeting, is under investigation. These data are consistent with a model in which a large collection of host proteins contribute to coupled nuclear import and subsequent integration in gene dense regions.

DECIPHERING THE CODE FOR RETROVIRAL INTEGRATION TARGET SITE SELECTION

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Upon cell invasion retroviruses generate a DNA copy of their RNA genome and integrate retroviral cDNA within host chromosomal DNA. These retrotransposition steps are essential and conserved features of retroviral replication. Integration occurs throughout the host cell genome but target site selection is not random and each subgroup of retrovirus is distinguished from the others by attraction to particular features on chromosomes. Despite extensive efforts to identify host factors that interact with retrovirion components, or chromosome primary structure and epigenetic markers predictive of integration, little is known about how integration sites are selected.

We attempted to identify markers predictive of retroviral integration by exploiting ChIPSeq (high-throughput sequencing technology combined with standard chromatin immunoprecipitation) and borrowing statistical concepts from the field of Information Retrieval. ChIPSeq produces genome-wide, chromosome-associated factor sitemaps of higher density than previously attainable. Precision-Recall methods developed for extracting information from highly skewed datasets were exploited to discriminate between individual chromatin-associated markers for retroviral integration and to combine the predictive power of multiple factors. Markers with F scores between 0.5 and 1.0 were considered to be associated with integration sites.

Currently, the best chromosomal markers for integration site selection are CpG islands and transcription start sites (TSS) associated with gammaretroviruses. 21 to 27% of MLV integration sites fall within 2 kB of these markers with F scores of 0.36 to 0.51. The F score for HIV-1 is 0.11. Then we compared ChIPSeq datasets for more than 70 factors with 12 retroviral integration data sets for HIV-1, MLV, HIV/MLV chimeras, PERV, XMRV, HTLV1, ASLV, and Foamy virus. When compared with MLV, PERV or XMRV integration sites, F scores >0.80 were observed with acetylation of H3 and H4 at several positions, methylation of H2AZ, H3K4 and K9, and STAT1. H3K4me3 vs MLV, for example, gave an F score of 0.84, while HIV-1 was only 0.21. The F score for a chimera in which HIV-1 gag and IN was replaced with those from MLV was 0.82. By combining highly specific markers, we generated a supermarker that, 1) predicts >70% of MLV integration site within 2 kB, 2) predicts the average number of proviruses within a specific chromosomal region of a specific cell line, 3) explain why some oncogenes are activated in certain cell and not in others.

THE HIV-1 INTASOME AND ITS INHIBITION: PROGRESS AND PROSPECTS FROM NEW STRUCTURE-BASED MODELS

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The intasome is the basic recombination unit of retroviral integration, comprised of the integrase (IN) protein and ends of the cDNA made by reverse transcription. Clinical IN strand transfer inhibitors (InSTIs) preferentially target the DNA bound form of IN as compared to the free protein, highlighting the critical requirement for detailed understanding of HIV-1 intasome structure and function. Though biochemical studies have identified a tetramer of HIV-1 IN as the active form of the enzyme, structural details of protein and DNA interactions are lacking.

The crystal structure of the related prototype foamy virus (PFV) intasome has been solved recently, revealing numerous details of the retroviral integration machinery. Structures of drug-bound PFV intasome complexes moreover shed significant light on the mechanism of InSTI action. Herein we present a model for the HIV-1 intasome built using the PFV structure as template. Analysis of critical IN-IN and IN-DNA interactions seen in the HIV-1 intasome and their comparison with previous results further validated our model. The roles of novel IN-DNA contacts are being assessed using a variety of biochemical assays.

Models for clinical InSTIs bound at the HIV-1 IN active site were also constructed and compared to results of previous studies. These data highlight key attributes of drug resistance and underscore interactions between halobenzyl moieties common to InSTIs and the invariant CA/GT residues at the end of the viral DNA substrate.

Our models highlight the structural basis for HIV-1 integration, define the mechanism of inhibitor action, and therefore should help to formulate new drugs to inhibit viruses resistant to first-in-class compounds.

HIV-1 INTASOME ASSEMBLY: FROM SINGLE END COMPLEX TO STABLE SYNAPTIC COMPLEX

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We have used atomic force microscopy to study stable complexes formed between HIV-1 integrase and viral DNA. A tetramer of integrase stably bridges a pair of viral DNA ends, consistent with previous analysis by gel electrophoresis. This intasome is highly stable to high ionic strength that strips more loosely associated integrase from internal regions of the viral DNA. We also observe tetramers of integrase associated with single viral DNA ends and time course experiments suggest that these Single End Complexes (SECs) are intermediates in intasome assembly. Strikingly, integrase tetramers are only observed in tight association with viral DNA ends and not at internal regions of the viral DNA. Dimers of integrase were never observed bound to viral DNA ends. The self-association properties of intasomes suggest that the integrase tetramer within the intasome is different from the integrase tetramer formed at high concentration in solution in the absence of viral DNA. Finally, the integration product remains tightly bound by the integrase tetramer, but the 3' ends of the target DNA in the complex are not restrained and are free to rotate resulting in relaxation of initially supercoiled target DNA. The crystal structure of the prototype foamy virus intasome[1] suggests that some integrase domains within the tetramer may be dispensable for intasome assembly. We are currently testing this hypothesis.

Reference:
1. Hare S, Gupta SS, Valkov E, Engelman A, Cherepanov P. Nature, published online 31 January 2010.

SEQUENTIAL INTERACTIONS OF VIRAL DNA AND LEDGF WITH HIV-1 INTEGRASE ARE ESSENTIAL FOR PRODUCTIVE CONCERTED INTEGRATION

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A tetrameric form of HIV-1 integrase (IN) stably associates with two viral DNA ends and catalyzes integration of the viral genome into the host chromosome, a process essential for retroviral replication. We and other groups have demonstrated that LEDGF also stabilizes a tetrameric form of IN. However, our mass spectrometric footprinting experiments and functional assays have suggested that IN tetramers formed in IN-viral DNA and IN-LEDGF complexes are not identical. For example, mass spectrometric footprinting experiments have indicated that IN undergoes significant conformational change upon interactions with DNA substrates but not with LEDGF. Furthermore, we and other groups have demonstrated that the pre-assembled IN-LEDGF complex is active in 3'-processing and single-end integration reactions but fails to catalyze the biologically relevant concerted integration of both ends. In contrast, pre-incubation of IN with vDNA and subsequent addition of LEDGF ensures effective concerted integration. In the present study we have examined a hypothesis that direct binding of LEDGF differentially modulates structural conformations and functions of the pre-formed IN-viral DNA complex and unliganded IN. To compare IN tetrameric forms in the IN-viral DNA and IN-LEDGF complexes we employed protein-protein FRET. For this, two separate preparations of IN were site-specifically labeled with donor and acceptor fluorophore. The results indicate that the IN tetramers adopt distinct conformations in the IN-LEDGF and IN-viral DNA complexes. In particular, the two IN active sites are positioned significantly closer together in the IN-viral DNA complex than the IN-LEDGF complex. FRET measurements suggest optimal organization of individual IN subunits within IN-viral DNA complex, while LEDGF stabilizes suboptimal conformation of IN tetramer. Furthermore, the order of addition experiments indicated that binding of LEDGF to the pre-formed IN-viral DNA complex allows maintenance of the fully functional conformation of the nucleoprotein complex, whereas preincubation of LEDGF and IN first and subsequent addition of viral DNA yield a distinct conformation of the IN tetramer which is defective for concerted integration. These findings provide new and important insight into the structural organization of IN in the functional ternary IN-viral DNA-LEDGF complex and highlight the importance of the sequential interactions of viral DNA and LEDGF for productive HIV-1 integration.

MECHANISM OF STRAND TRANSFER INHIBITORS: IDENTIFICATION OF A MAJOR NOVEL IN-SINGLE DNA-INHIBITOR COMPLEX.

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Integration of HIV-1 DNA into chromosomes by the viral integrase (IN) is essential for virus replication. Upon reverse transcription, a preintegration complex (PIC) is formed wherein IN removes two nucleotides from the 3'-OH blunt-ends of linear cDNA. After nuclear transport, IN inserts the two recessed DNA ends into chromosomes via a concerted integration mechanism. In-vitro, IN juxtaposes two viral DNA ends forming the synaptic complex (SC), a transient intermediate in the concerted integration pathway. Here, we show Raltegravir (RAL) and other strand transfer inhibitors (STI) possessing diverse structures bind to and inactivate SC. The inactivated or trapped SC is unable to bind target DNA thereby inhibiting the formation of concerted integration products. Efficiency of a particular inhibitor to form trapped SC correlates with its potency to inhibit concerted integration.

We detected a new major IN-single viral DNA-inhibitor complex on native agarose above 200 nM STI, termed inhibitor-single DNA (ISD) complex. IN dimers in ISD complex bind to the terminal ~30 bp on U5 and U3 DNA ends. Formation of ISD was time, temperature, and STI concentration dependent. With RAL, MK-2048, and diketo acid L-841,411, ~20-30% of the input DNA substrate was incorporated into the ISD complex. Most of other seven STI investigated including Elvitegravir weakly produced ISD (≤5% of the input DNA). RAL resistant mutant N155H weakly formed the ISD complex with RAL at 25% level of wild type IN which correlates with the ~3 fold increase in the IC_{50} value for inhibition of concerted integration by N155H. The ISD complex may be valuable in the biochemical evaluation of newly identified STI, drug-resistant IN mutants, and atomic structural analysis.

CHARACTERIZATION OF THE DYNAMIC INTERPLAY BETWEEN HIV-1 INTEGRASE AND ITS CELLULAR COFACTORS DURING LYMPHOCYTE INFECTION

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Exploring the dynamic interplay between HIV-1 and host cell factors is essential to understand the molecular mechanisms underlying diverse physiological aspects of the HIV-1 life cycle. During early steps, viral components, as part of the pre-integration complex (PIC), have to travel from the plasma membrane and then cross the nuclear pore to finally integrate viral cDNA into the host cell genome. This latter event is controlled by the viral enzyme integrase (IN), which has to interact with both viral and cellular proteins to achieve integration. Because IN is one of the components that remains constantly associated with PIC until reaching its chromosomal target it is particularly suited for studying interactions between PIC and cellular factors.
In this study, we developed a biochemical approach to further explore the dynamics of interactions that IN engaged with its cellular and viral partners at early time points post-infection. We took advantage of the HIV-1 Bru virus carrying an IN tagged with an HA epitope to purify and characterize IN complexes during the course of T-lymphocyte infection. At two hours post-infection (h.p.i.), the majority of IN co-eluted with viral DNA within a high molecular mass (IN-HMM) complex, as analyzed by gel-filtration chromatography. This complex is dramatically reduced at later time points, while several low molecular mass IN complexes (IN-LMM) are still detectable. These IN-LMM complexes were found to co-fractionate with cellular cofactors LEDGF/p75, VBP1 and TNPO3. Furthermore, interaction between IN and LEDGF/p75, VBP1 and TNPO3 could also be detected at 6 h.p.i. Overall, our results indicate a dynamic recruitment of cellular cofactors by IN during the early steps of HIV replication.

THE ROLE OF ENDOGENOUS RETROVIRUS EXPRESSION AS A MARKER FOR CELLULAR TRANSFORMATION

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Human endogenous retroviruses (HERVs) result from viral infections in the germ line and constitute about 8% of the human genome. The HERV-K (HML-2) group includes the most recently integrated human proviruses, some of which are expressed in normal and cancer tissue. Examples of HERV-K expression, ranging from RNA transcripts to virus particles visible in electron micrographs, have been observed in mammary tumors. Since HERVs represent a significant fraction of the human genome, endogenous proviruses might play an important role in the regulation of cellular gene expression leading to tumor formation. Identification of HERV loci may also provide useful indicators of altered gene expression from various cellular DNA regions as well as understanding the underlying mechanisms of tumorigenesis.

To assess these possibilities, we have examined HERV-K (HML2) expression patterns in a set of breast cancer cell lines, as well as primary mammary epithelial and tumor cells. We have identified a subset of HML-2 proviruses that is expressed uniquely in tumor cells of various types, but not in normal mammary epithelium. From these proviruses, we have analyzed published microarray data to determine whether their expression is linked to expression of genes implicated in tumorigenesis through local changes in chromatin structure or through shared promoter elements. Identification of provirus-linked genes that are significantly upregulated in tumor samples which will provide important insights into the mechanism of tumorigenesis and may identify putative anti-tumor targets. Finally, we have shown that envelope (Env) proteins encoded by HERV-K proviruses are expressed in all tumor cell lines examined but not in normal lines, enhancing the prospect of using Env as a possible biomarker for tumorigenesis.

IDENTIFICATION OF HOST FACTORS THAT INITIATE AND MAINTAIN RETROVIRAL EPIGENETIC SILENCING

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Epigenetic silencing of retroviral DNA may occur through either stochastic or active processes. Stochastic processes include integration site position effects, while active processes may reflect a cellular antiviral response. We have been investigating the mechanisms that underlie the rapid, and high frequency epigenetic silencing of avian sarcoma virus in human cells, using RCAS/RCAN vectors that encode GFP reporter genes under control of the LTR or heterologous promoters. We show that epigenetic silencing proceeds through two steps: initiation and maintenance. Initiation appears to be an active process, whereby host factors engage the incoming viral DNA. We had previously discovered such an inhibitory role for the ubiquitous host protein Daxx, a transcriptional repressor. Daxx is now recognized to have broad antiviral roles, with repressive activities mediated via its binding partners, histone deacetylases (HDACs) and DNA methyl transferases (DNMTs). Our recent studies address the roles of these factors, and show that proviral DNA methylation occurs very rapidly, post-infection. Furthermore, knockdown of Daxx prior to infection results in increased GFP expression, consistent with its proposed repressive role.

To identify the cellular factors that maintain long term silencing, we previously developed an siRNA-based screening approach, in which knockdown of specific silencing factors results in viral GFP reporter reactivation. We identified functional roles for DNMT3A, as well as the histone methyltransferases KMT1E and KMT5C, which place the H3K9me3 and H4K20me3 repressive marks, respectively. Our recent findings indicate that these histone repressive marks, as well as CpG DNA methylation are widespread over the integrated proviral DNA. Our current data suggest a model in which the silencing machinery is recruited to viral DNA to initiate and maintain a heterochromatic state. This experimental system provides a robust approach for identifying cellular epigenetic silencing factors.

CELLULAR TRANSCRIPTION FACTOR ZASC1 STIMULATES HIV-1 TAT ACTIVITY.

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Previously we reported the results of a somatic cell genetic screen of insertionally mutagenized Chinese hamster ovary (CHO-K1) cells that identified ZASC1 as a novel transcriptional regulator of MLV transcription. ZASC1 was shown to be a sequence-specific DNA binding protein with three highly similar binding sites located within the MLV U3 promoter. Sequence homology analysis revealed that the HIV-1 promoter contained a highly conserved ZASC1 binding site located just upstream of the TAR element. EMSA analysis confirmed that ZASC1 bound the HIV-1 promoter and infection with HIV-1 vectors containing mutations in the U3 region that disrupt ZASC1 binding led to impairment of proviral transcription in newly infected CHO-K1 cells or human Jurkat T cells. The role of ZASC1 in HIV-1 infection was confirmed using dominant negative ZASC1 mutants and gene-specific shRNAs that reduced the level of ZASC1 protein prior to virus challenge. Due to the close proximity of the ZASC1 binding site to the TAR element, we examined the potential role of ZASC1 in TAT mediated activation of transcription. In transient transfection assays, TAT mediated stimulation of promoters lacking ZASC1 binding sites was significantly lower than that seen with WT promoters. In contrast to ZASC1 effects on the MLV promoter, expressing exogenous ZASC1 did not, by itself, stimulate the HIV-1 promoter. However, TAT and ZASC1 co-expression significantly increased TAT activation, and this stimulation of TAT activity was dependent upon the ZASC1 binding sites in the viral promoter. Mammalian two-hybrid and co-immunoprecipitation experiments demonstrated a physical interaction between ZASC1 and TAT. These data demonstrate that ZASC1 is a new transcriptional regulator of HIV-1 gene expression that binds to DNA sequences in the HIV-1 promoter, interacts with TAT and stimulates TAT-mediated activation of viral transcription.

NUCLEAR RETENTION OF HIV-1 PRE-MRNA THROUGH PSF/P54NRB AND MATRIN3 IS REQUIRED FOR HIV-1 REV

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Nuclear retention of RNA is implicated in the regulation of several pathways of gene expression. A good model of functional nuclear RNA retention comes from the human immunodeficiency virus type 1 (HIV-1) where the generation of infectious virions requires the synthesis of two classes of viral transcripts: the spliced and the non-terminally spliced HIV RNA. The former is efficiently processed and exported to the cytoplasm. The latter remains unspliced or partially spliced and it is retained in the nucleus by unknown mechanism until the viral Rev protein mediates its export for the expression of structural proteins and for the production of full-length genomic RNAs.
A major mechanism of nuclear RNA retention involves hyperediting by adenosine deamination. We show that hyperediting is not obligatory for retention of viral unspliced RNA and we characterize an alternative pathway.
To this end we explored the proteome associated with the unspliced HIV-1 RNAs that are retained in the nucleus until the viral Rev proteins mediate their export. We demonstrate that the viral unspliced RNA is associated with the host factors PSF, $p54^{nrb}$ and MATR3. PSF/$p54^{nrb}$ complex binds the viral RNA co-transcriptionally but MATR3 defines a subnuclear compartment where the viral RNA is delivered. This pathway is required for the activity of Rev on unspliced HIV-1 RNA. Interestingly PSF/$p54^{nrb}$ binding and localization to MATR3 foci occur independently of RNA hyperediting.
Our findings reveal a novel cellular mechanism of nuclear RNA retention that is hijacked by a virus.

MOUSE MAMMARY TUMOR VIRUS REM PROTEIN REQUIRES PROCESSING BY SIGNAL PEPTIDASE AND RETROTRANSLOCATION FOR NUCLEAR FUNCTION

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Mouse mammary tumor virus (MMTV) is a complex murine retrovirus that encodes an HIV Rev-like export protein, Rem, from a doubly spliced version of envelope (*env*) mRNA. Previously, the N-terminal 98 amino acids of Rem, which is identical to Env signal peptide (SP), and full-length Rem were shown to be functional in a reporter assay and localized to the nucleolus. The reporter assay is based on the *Renilla* luciferase gene, which has been inserted into the 3' end of the MMTV genome. Luciferase activity is responsive to Rem or Env expression and requires a functional nuclear localization sequence in the SP from either protein. Rem-responsiveness also requires a Rem-responsive element (RmRE) on the reporter vector. MMTV-infected cells or cells transfected with *rem* or envelope cDNAs express SP. Signal peptides generally function to direct translation of viral or cellular glycoproteins to the endoplasmic reticulum (ER). Signal peptidase, a protease located in the ER lumen, then cleaves the signal peptide, which is believed to be degraded by cytosolic proteasomes. Mutations that block Rem or Env cleavage by signal peptidase eliminate both detectable SP and functional activity in reporter assays. Fluorescence microscopy revealed that GFP-tagged cleavage site mutants are unstable and lack fluorescence compared to GFP-tagged wild-type Rem, suggesting improper folding. Uncleaved Rem is partially glycosylated, yet mutations in both glycosylation sites within the C-terminus prevented Rem function. Proteasome inhibitors allowed accumulation of uncleaved Rem relative to SP and increased reporter activity, consistent with SP retrotranslocation and proteasomal escape prior to nuclear entry. Expression of a dominant-negative p97 ATPase did not alter levels of unprocessed Rem and SP, but decreased reporter activity, suggesting p97-dependent retrotranslocation of SP. Our results provide the first example of a SP that is processed by signal peptidase and retrotranslocated to allow nuclear localization and function.

RNA CAP HYPERMETHYLTRANSFERASE AND HIV-1 GENE EXPRESSION

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Human Immunodeficiency Virus Type 1 uses a complex regulatory mechanism, involving both viral and cellular genes, to control gene expression. In case of HIV-1 a single transcript in its unspliced and spliced forms directs the synthesis of all viral proteins. Normally the nuclear export of intron containing / unspliced RNA transcripts is inhibited in all eukaryotic cells. However, post-transcriptional regulatory systems involving viral elements like Rev/RRE and CTE have been shown to overcome the restrictions of cellular RNA export mechanisms. HIV-1 has evolved the viral Rev protein to overcome this restriction for viral transcripts. RNA capping and cap-methylation occur co-transcriptionally at the start of transcription and 5'- mRNA capping has important consequences for events such as pre-mRNA synthesis / splicing, RNA cytoplasmic transport, mRNA translation and turnover. In eukaryotes, a 7-methylguanosine (m7G) cap is added to newly transcribed RNA polymerase II (RNAP II) transcripts. A subset of RNAP II transcribed cellular RNAs, including small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and telomerase RNA, are additionally hypermethylated at the exocyclic N2 of the guanosine cap. These RNAs, different from m7G-RNA, are exported from the nucleus through the CRM-1 pathway. The CRM-1 pathway is also used by HIV-1 to export Rev/RRE-dependant RNAs into the cytoplasm and previously, it was considered that the HIV-1 Rev protein and the cis-acting viral RRE were sufficient for dictating export specificity through CRM-1. Here, we report that HIV-1 Rev/RRE-dependant RNA, like snRNA and snoRNA, has a trimethylguanosine (TMG) cap. We identify the trimethylating methyltransferase as the human PIMT (PPAR-interacting protein with methyltransferase domain) protein and show that PIMT-mediated TMG modification specifies CRM-1 export of Rev/RRE-dependent RNA.

RNA HELICASE A RESIDUES NECESSARY FOR SPECIFIC RECOGNITION AND TRANSLATION OF AVIAN AND HUMAN RETROVIRAL RNAS

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Retroviruses rely on host RNA binding proteins to modulate various steps in their replication. Many retroviruses require Dhx9/RNA helicase A (RHA) for efficient virion production. In HIV-1, RHA is a necessary host factor for efficient translation of viral mRNA, and RHA assembled into progeny virions promotes infectivity of these virions. Biophysical and genetic approaches defined features of RHA necessary for selective interaction with retroviral target RNA. Site-directed mutagenesis determined that helicase activity associated with the central DEIH helicase core is necessary for HIV-1 and HTLV-1 translation activity; the C-terminal RGG-rich domain is necessary for nuclear localization of RHA and protein-protein interactions necessary for translation activity, but not RNA recognition. The N-terminal RNA binding domain directs specific recognition of retroviral target RNA. Substitution mutations identified critical N-terminal Lysine residues; their mutation is sufficient to eliminate target RNA affinity. Mutation or deletion of N-terminus eliminates RHA translation activity. Furthermore, expression of the RHA N-terminus is sufficient to block RHA-dependent translation activity. Our results support the expectation that host modification of RHA is sufficient to downregulate RHA-dependent translation of viral and host genes. The results suggest RHA is the nexus of a newly appreciated antiviral response mechanism.

SPECIFIC REPRESSION OF MOLONEY MURINE LEUKEMIA VIRUS TRANSCRIPTION SUPPORTS THE EARLY BISECTION IN VIRAL UNSPLICED RNA INTO MRNA AND GENOME FATES.

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Unspliced retroviral RNAs function as genomes (gRNA) for packaging and mRNAs for translation. Where and when gRNAs initially form dimer partner associations or are recruited for packaging likely differs among retroviruses. For Moloney murine leukemia virus (MLV), previous findings with actinomycin D treated cells suggest that MLV unspliced RNAs sort into non-equilibrating pools of gRNAs and mRNAs. Prior work from our lab and others on the randomness of gRNA dimer populations suggests that unlike HIV-1, MLV gRNA dimer partner selection may occur before RNAs exit the nucleus, suggesting that the bisection in gRNA and mRNA fates occurs before nuclear egress. Here, tetracycline-regulated MLV transcription was used to repress MLV transcription in a more targeted fashion in order to re-address the two pool phenotype of MLV unspliced RNA. Specific repression of MLV transcription led to a more rapid decline in gRNA packaging than virion production which is consistent with earlier findings where transcription was inhibited globally with actinomycin D. Additionally, packaged gRNA dimer populations were analyzed at various time points after both specific and general transcription inhibition. The results indicated that transcription inhibition led to increased randomness in gRNA dimer partner associations and a parallel increase in recombination under both conditions, albeit with residual biases consistent with an ongoing partitioning between mRNAs and genomes. These data suggest that the small population of heterodimers in MLV co-expression studies arise from a subset of gRNAs that escape early dimer partner associations and form dimer associations more randomly later. Together, our observations suggest that MLV unspliced RNA fates are determined early in the host cell nucleus and support the early separation of MLV unspliced RNAs into two largely non-equilibrating pools.

SUPPRESSION OF NONSENSE-MEDIATED DECAY BY RETROVIRAL RECODING ELEMENTS

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An emerging unified model of nonsense-mediated decay (NMD) suggests that long 3' untranslated regions (3' UTRs) render mRNAs susceptible to NMD in mammalian cells. Retroviruses, which invariably express mRNAs containing 3' UTRs spanning several kilobases, have a complex relationship with the NMD surveillance machinery. For example, avian retroviruses have evolved RNA elements that suppress degradation of messages harboring premature termination codons, while it has been reported that the core NMD factor Upf1 is a positive regulator of HIV RNA stability.

We are using affinity purification of mRNP complexes containing tagged RNAs to identify proteins associated with the 3' UTRs of HIV mRNAs. In the course of these experiments, we observed enrichment of Upf1 on transcripts containing HIV 3'LTR sequences. We find that the association of Upf1 with HIV 3'LTR-containing mRNAs is not sequence-specific but instead correlates with UTR length. To better understand the functional significance of Upf1 recruitment to transcripts with long UTRs, we analyzed Upf1 binding to model mRNAs containing 3'UTRs known to be either susceptible or resistant to NMD. We observed some Upf1 association with all transcripts tested, but mRNAs with 3' UTRs known to undergo Upf1-dependent decay co-purified substantially higher levels of Upf1 protein than those resistant to NMD, in a manner independent of ongoing translation and functional NMD. Together, these findings suggest that nonspecific RNA binding by Upf1 may allow it to act as an intrinsic sensor of UTR length in the process of premature termination codon surveillance.

The Moloney murine leukemia virus (M-MLV) readthrough-promoting pseudoknot causes misincorporation of an amino acid at the gag termination codon at 5-10% frequency. Using the M-MLV pseudoknot to modulate translation termination at an NMD-triggering premature termination codon, we find that readthrough disrupts steady-state Upf1 association with transcripts containing long UTRs. Surprisingly, we also demonstrate that either readthrough or frameshifting by retroviral RNA structures can robustly inhibit NMD. In fact, even M-MLV pseudoknot variants causing levels of readthrough insufficient to disrupt Upf1 association with long UTR-containing RNAs can stabilize NMD targets. By modulating readthrough efficiency, we are thus able to separate Upf1 recognition of long UTR-containing RNAs from downstream RNA decay, giving valuable insight into the in vivo dynamics of transcript surveillance and decay by the NMD pathway. In addition, this work uncovers a potential role for recoding elements in the evasion of NMD by retroviruses.

IMAGING RSV GAG-VIRAL RNA INTERACTIONS IN THE NUCLEI OF LIVING CELLS

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The Rous sarcoma virus (RSV) Gag protein coordinates the assembly and release of virus particles from the plasma membrane. Previously, we demonstrated that RSV Gag undergoes transient nuclear localization prior to membrane binding. Nucleocytoplasmic shuttling of Gag is intrinsic to the virus assembly pathway. If Gag bypasses the nuclear trafficking step, virion incorporation of genomic RNA (gRNA) is severely impaired, although re-establishing Gag nuclear transport restores gRNA encapsidation. Taken together, these results strongly suggest that Gag nuclear trafficking is involved in the recognition and selective encapsidation of RSV gRNA.

To directly test whether we could visualize Gag-gRNA interactions within the nucleus, we adapted the MS2-phage labeling system to tag the viral RNA with a fluorophore. We engineered an RSV provirus containing 24 repeats of MS2-RNA stem loops (RC-24x). The MS2 coat protein fused to YFP (MS2-YFP) recognizes this RNA stem loop structure and selectively binds it, allowing the detection of gRNA in live cells. Incorporation of 24 copies of the MS2 stem loops into the RSV genome did not disrupt productive infection in avian cells.

To visualize where Gag interacts with the gRNA in living cells, we expressed Gag-CFP in trans. MS2-YFP, which harbors a strong nuclear localization signal, remains diffuse throughout the nucleus when co-expressed with an RSV provirus lacking the MS2 loops (RC-wt), and little MS2-YFP was seen outside the nucleus. When the RC-24x provirus was expressed, MS2-YFP formed discrete puncta in the nucleoplasm as well as foci just outside the nucleus, in the cytoplasm, and at the plasma membrane that colocalized with Gag.

To study Gag-gRNA interactions in the nucleus, cells expressing RC-24x or RC-wt, MS2-YFP, and Gag-CFP were treated with leptomycin B (LMB) to inhibit Gag nuclear export. In LMB treated cells, MS2-YFP formed larger intranuclear puncta that were localized at or near foci of Gag-CFP with RC-24x but not RC-wt. Colocalization of Gag with the MS2-tagged gRNA suggests that RSV Gag binds its genome in the nucleus. To better characterize these intranuclear binding events, we will use a nuclear-export deficient mutant of Gag (L219A) to study the mobility of Gag-gRNA nuclear foci. Furthermore, we will use time-lapse experiments to follow the MS2-tagged gRNA puncta from the nucleus, through the cytoplasm, and to the plasma membrane for particle release.

DISSECTING THE MECHANISMS OF HIV-2 RNA PACKAGING

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We recently developed a system to directly examine the RNA content of individual HIV-1 particles, using fluorescent protein (FP)-tagged RNA-binding proteins that specifically recognize stem-loop structures engineered into the viral genome. We have now applied a similar strategy to study the mechanisms of HIV-2 RNA packaging, which has been proposed to be different from that of HIV-1; specifically, HIV-2 Gag packages the RNA in cis and only RNA that encodes full-length functional Gag can be efficiently packaged.

To delineate the efficiency of HIV-2 genome encapsidation and explore the mechanisms of HIV-2 RNA packaging, we modified HIV-2 genomes, coexpressed these genomes with FP-tagged RNA-binding proteins, harvested virus-like particles, and directly examined the RNA content of each particle by fluorescence microscopy. We observed that >90% of the HIV-2 particles contained full-length viral RNA. Using two different tagged RNA-binding proteins, we found that heterozygous particles, which contained RNAs from two different viruses, were generated efficiently. Furthermore, the ratio of the heterozygous particles in the viral population could be altered by changing the dimerization initiation signal (DIS) in the 5′ untranslated region, suggesting that HIV-2 Gag packages one dimeric RNA rather than two monomeric RNAs.

To further explore whether HIV-2 packages RNA in cis, we generated gag mutants that do not encode functional proteins and used a competition assay to compare the packaging efficiencies of RNAs from viruses encoding wild-type or mutant Gag. Our preliminary results showed that mutant Gag-encoding RNAs were efficiently packaged into particles.

Together, these findings indicated that HIV-2 RNA is encapsidated into particles at a high efficiency, HIV-2 Gag packages dimeric RNA, and efficient HIV-2 packaging does not require RNA that encodes full-length functional Gag.

PREFERRED GAG BINDING STIES ARE CRUCIAL PACKAGING SIGNAL ELEMENTS IN MLV GENOMIC RNA

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All retroviral genomic RNAs contain a cis-acting packaging signal, termed ψ, but the mechanism by which ψ-containing RNAs are selectively packaged into nascent virions is not understood. Ψ could contain a high-affinity binding site for Gag; however, it is equally possible that ψ directs the RNA to a cellular compartment where it will encounter Gag before Gag can interact with cellular mRNAs (which can also be packaged). Retroviral RNAs are packaged as dimers and it has been suggested that dimerization is essential for selective packaging. Using the SHAPE method of high-throughput analysis of RNA secondary structure, we have studied the structure of Moloney murine leukemia virus (MLV) RNA within the authentic virus particle and compared this structure to that of naked viral RNA. We also analyzed the binding of recombinant MLV Gag protein (the structural protein of the virus) and NC protein (the nucleic acid-binding protein within mature MLV particles) to dimeric RNA in vitro. These experiments indicate that both Gag and NC bind specifically to the sequence UCUG-UR-UCUG and interact most strongly with the first U and final G in each tandemly repeated UCUG element. The context surrounding these ten nucleotides is also important, as highest affinity binding requires the presence of base-paired regions flanking this motif. Two of these motifs are partially base-paired in monomeric MLV RNA, but are unpaired in dimeric RNA. We tested the possibility that this high-affinity binding structure, specifically created upon dimerization of MLV RNA, is important in packaging. We found that a mutant in which all 4 of the G residues in the two motifs are replaced by A nucleotides is packaged <1% as efficiently as the native sequence. Thus, this motif is a crucial element in ψ. To our knowledge, this is the first time that alteration of a small number of bases has had such a dramatic effect on the packaging of a retroviral RNA. The results support the general hypothesis that ψ is a high-affinity binding structure for the homologous Gag protein.

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CELLULAR FACTORS THAT RESTORE HIV-1 ASSEMBLY IN MOUSE CELLS

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HIV-1 assembles inefficiently in murine cells, reflecting weak expression of the Gag/Gag-Pol structural proteins and a cellular defect in Gag targeting to the plasma membrane. Mouse-human cell fusions or hybrids can provide for infectious virus production, suggesting that murine cells lack one or more human factors necessary for HIV-1 assembly. We previously demonstrated that HIV particle production in murine cells is restored after replacing the Rev-response element (RRE) in Gag/Gag-Pol transcripts with four copies of the constitutive transport element (CTE) from Mason-Pfizer monkey virus. The RRE confers nuclear export of unspliced and partially spliced viral transcripts using the HIV Rev/Crm1-dependent pathway, while the CTE directs export through the NXF1/NXT pathway used by the bulk of cellular mRNAs. This result suggests a mechanistic link between transport of the viral genome, serving as the mRNA encoding Gag/Gag-Pol, and Gag assembly competency. Subsequently, we have designed RNA transcripts encoding Gag/Gag-Pol and various Gag mutants under the control of different nuclear export elements and have used these to study the vertical integration of HIV-1 gene expression as well as to identify and characterize human cellular factors that may contribute to the HIV-1 assembly pathway. These studies have identified multiple cellular factors that, when expressed transiently, markedly improve Gag/Gag-Pol expression and virus particle production. A subset of these factors, most notably members of the SR family of RNA binding proteins, result in robust increases to Gag/Gag-Pol expression specifically from HIV-1 RNAs. However, when controlled for expression level, SR proteins do not overcome the defect to Gag assembly competency. By contrast, other factors such as Sam68 both boost Gag/Gag-Pol expression and improve Gag assembly competency in murine cells. We are currently asking if these proteins may contribute to restoring virus replication in murine cells

HOST CELL FACTORS IN RETROVIRUS ASSEMBLY AND RELEASE

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Retroviral assembly and release are highly coordinated events mediated via specific interactions between the viral Gag protein and the host cell machinery. Previous work from our lab demonstrated the involvement of Golgi-localized, γ-ear-containing, Arf-binding proteins (GGAs) and ADP ribosylation factor (Arf) proteins in HIV-1 assembly and release. In this study, we identified the cellular protein growth arrest-specific 7 (Gas7) as a novel candidate host cofactor in HIV-1 replication. Gas7, a Pombe cdc15 homology (PCH) protein harboring functional determinants like F-BAR, WW, and SH3-domains, interacts not only with HIV-1 Gag but also with cellular cofactors that have been implicated in the late stages of retroviral replication (e.g., GGA1, Tsg101, and SH3GL1). The F-BAR domain of Gas7 is known to bind to actin and induce plasma membrane outgrowths, while the WW-domain is reported to be essential for Gas7-mediated microtubular maintenance. We observe that exogenously expressed, full-length Gas7b is incorporated into virions and subsequently processed by the viral protease. In contrast, the Gag-binding F-BAR domain by itself is excluded from virions. This apparent WW-domain dependence of virion incorporation is further supported by our observation that the Gas7-binding protein PSTPIP1, a WW-domain deficient but F-BAR domain-harboring member of the same family, is excluded from virions. Bimolecular fluorescence complementation (BiFC) assays, in addition to providing evidence for direct interaction of Gas7 with HIV-1 Gag and GGA1, enabled visualization of plasma membrane-localized actin tail-like structures resulting from BiFC between Gas7 and HIV-1 Gag. We also obtained evidence by BiFC that Gas7 binds EIAV Gag in living cells. Interestingly, we observed an interaction between Gas7 and the WASP-interacting protein (WIP) in yeast two-hybrid assays, and a recent report demonstrated a direct interaction between Gas7 and N-WASP via the WW-domain. These findings imply a role for WIP:WASP:Arp2/3 pathway in Gas7-mediated membrane remodeling events that could potentially modulate retroviral assembly and release at the plasma membrane. The reported enhancement of Gas7 expression levels in polarized monocyte-derived macrophages and the generation of tubule-like structures upon Gas7 dimerization in our BiFC assays also suggest additional roles for Gas7 such as in intercellular transfer of retroviruses. Data deciphering the functional role of Gas7 and its cellular partners in retroviral replication will be presented.

LIVE-CELL IMAGING OF SKD1 RECRUITMENT AND DYNAMICS DURING HIV-1 BUDDING

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In the last years, biochemical and structural studies have indicated the crucial involvement of host cell ESCRT components during the HIV-1 budding process. The AAA ATPase SKD1 (Vps4) acts in the final stage of ESCRT function, resolving the ESCRT III complex in order to recycle its components.

Using fluorescently labelled SKD1, we have directly visualized the dynamics of SKD1 activity during the Gag assembly and release process by fluorescence live-cell imaging over 1-2 hours at 37°C. SKD1eGFP coexpressed with GagmCherry and Gagwt showed fluorescence bursts at the plasma membrane of living cells, colocalizing with viral assembly sites. Specific recruitment of SKD1 to Gag assembly sites was verified by the absence of SKD1 bursts if no Gag or Gag late- mutants were expressed. Moreover, the number of bursts increased with the number of Gag assembly sites connecting those bursts to SKD1 activity. Coexpression of the SKD1E228Q mutant, which prevents ATP hydrolysis and has been reported to arrest SKD1 in its multimeric ring structure, resulted in frozen bursts at the plasma membrane, largely not colocalizing with Gag assembly sites. By tracking individual assembly sites, we could establish the timing of the SKD1 interaction with respect to the three phases of HIV-1 budding established in earlier studies (PLOS Pathogen 5(11), 2009). SKD1 bursts appeared mainly at the beginning of phase II when the Gag lattice had been completed and these bursts lasted in the order of one minute. On average, less than one burst was detected per assembly site, indicating that not every assembly site interacts with SKD1 (at least on the timescale of the two-hour measurement). Those assembly sites that did interact with SKD1 showed either a single burst or a low number of bursts. Mobility of Gag particles often increased or they disappeared on a delayed time scale after SKD1 bursts indicating that SKD1 recruitment is not immediately followed by virus release.

These studies provide direct information regarding the interaction of the cellular component SKD1 with nascent HIV-1 assembly sites. Such live-cell imaging experiments provide great promise in elucidating more details of the viral life cycle.

DETERMINANTS OF HIV-1 CAPSID ASSEMBLY

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The HIV-1 capsid (CA) protein assembles mature cylindrical and conical cores during viral morphogenesis *in vivo*, and can be induced to assemble cones, spheres and cylinders (tubes) *in vitro*. CA proteins are composed of N-terminal domains (NTDs), which have an amino-terminal β-hairpin and seven α helices (H1-H7); and a C-terminal domain (CTD), which folds to yield a short 3_{10}-helix followed by four α helices (H8-H11). Within capsid assembly products, CA proteins organize as hexamers, featuring NTD-NTD contacts mediated through H1-H3, intermolecular contacts between NTD H4 and a CTD groove, and interlinking of hexamers via the CTD dimer interface (H9). We have examined the determinants of HIV-1 capsid assembly *in vivo* and *in vitro*. Not surprisingly, we have found that perturbation of NTD β-hairpin, H1 and H2 residues impairs tube assembly; and that while deletion of the NTD cyclophilin loop (between H4 and H5) fosters assembly *in vitro*, the mutation yields assembly competent, but non-infectious virus. Interestingly, we also have identified mutations in H6 that alter assembly morphologies, suggesting a β-hairpin-H6 interaction that regulates core morphogenesis. Additional experiments have led to the identification of second site revertants of NTD H4 mutations that map to the CTD, but not directly to residues at the CTD groove. These results support a more complicated model of intermolecular NTD-CTD interactions than previously reported. Yet other experiments show that capsid assembly can be triggered via the addition of low concentrations of RNA and capsid-nucleocapsid (CANC) proteins, demonstrating a two step nucleation-growth mechanism for core assembly. Finally, in examining the effects of peptide inhibitors of capsid tube assembly, we have found that whereas the known inhibitor CAI blocks tube assembly, and can dismantle assembled tubes; two other peptides substantially alter the morphologies of the CA tubes. Our results identify new CA assembly determinants that may be targeted for the inhibition of HIV.

ASSEMBLY AND REPLICATION OF HIV-1 IN T CELLS WITH LOW $PI(4,5)P_2$ LEVELS

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HIV-1 Gag assembles into virus particles predominantly at the plasma membrane (PM). The MA domain promotes binding to the PM via myristic acid moiety at the N terminus and the highly basic region (HBR) (the residues 17-31) in MA. In addition, MA is involved in envelope incorporation into virions and early post-entry process, both of which affect infectivity of progeny virions. Previously, we observed that phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] interacts with HBR and that this interaction is essential for Gag binding to the PM and virus release in HeLa cells. However, it is still unknown whether $PI(4,5)P_2$ is similarly required in T cells.

In this study, we demonstrate that $PI(4,5)P_2$ also facilitates Gag binding to the PM and efficient virus release in T cells. In the presence of active polyphosphoinositide 5-phosphatase IV (5ptaseIV) that depletes cellular $PI(4,5)P_2$, Gag localization at the PM was reduced, and instead, mislocalized to intracellular compartments or remained in the cytosol. Consistent with this observation, virus particle production was reduced in A3.01 T cells expressing 5ptaseIV. Serial passage of HIV-1 in an A3.01 clone that expresses 5ptaseIV upon infection yielded an adapted mutant virus that has an L to R change at the MA residue 74 (74LR). The 74LR mutation accelerated the virus replication in T cells expressing 5ptaseIV relative to WT. However, single-cycle experiments revealed that the 74LR mutant was less efficient in virus release from parental A3.01 than WT, and in 5ptaseIV-expressing clones, release of the 74LR mutant is not more efficient than that of WT. Thus, accelerated replication of the 74LR mutant relative to WT in 5ptaseIV-expressing clones is not likely due to improved virus release. Instead, we found that the 74LR mutation increased the virus infectivity and compensated for the lack of $PI(4,5)P_2$-dependent virus release enhancement. Altogether, these results indicate that $PI(4,5)P_2$ enhances Gag-membrane binding, Gag targeting to the PM and virus release efficiency in T cells, which in turn likely promotes efficient virus spread in T cell cultures. In the absence of $PI(4,5)P_2$, however, the inefficient virus particle production can be compensated for by a mutation that enhances the virus infectivity.

THE ESCRT-ASSOCIATED ALIX PROTEIN RECRUITS THE UBIQUITIN LIGASE NEDD4-1 TO FACILITATE HIV-1 RELEASE THROUGH THE LYPXnL LATE DOMAIN MOTIF

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HIV-1 Gag contains two late (L) domains: PTAP and LYPXnL that recruit members of the host's fission machinery (ESCRT) to facilitate HIV-1 release. Other retroviruses gain access to the ESCRT pathway by utilizing a PPXY-type L domain that interacts with cellular Nedd4-like ubiquitin ligases. Despite the absence of a PPXY motif in HIV-1 Gag, interactions with Nedd4-2 were recently shown to stimulate HIV-1 release. Here we show that the ubiquitin ligase Nedd4-1 corrected release defects resulting from the disruption of PTAP (PTAP-), suggesting that HIV-1 Gag might also recruit Nedd4-1 to facilitate virus release. Notably, Nedd4-1 remediation of HIV-1 PTAP- budding defects is independent of cellular Tsg101, suggesting that Nedd4-1 function in HIV-1 release does not involve ESCRT-I components and is therefore distinct from that of Nedd4-2. Consistent with this finding, deletion of the p6 region decreased Nedd4-1-Gag interaction, and disruption of the LYPXnL motif eliminated Nedd4-1-mediated restoration of HIV-1 PTAP-. This result indicated that both Nedd4-1 interaction with Gag and function in virus release occur through the Alix-binding LYPXnL motif. Furthermore, mutations of basic residues located in the NC domain of Gag that are critical for Alix's facilitation of HIV-1 release, also disrupted release mediated by Nedd4-1, further confirming a Nedd4-1-Alix functional interdependence. In fact we found that Nedd4-1 binds Alix in both immunoprecipitation and yeast-two hybrid assays. In addition, Alix rescue of HIV-1 PTAP- required cellular Nedd4-1 supporting the notion that Alix must recruit Nedd4-1 to promote HIV-1 production. To promote virus release, Nedd4-1 also requires its catalytic activity, and RNAi knockdown of cellular Nedd4-1 inhibited Alix ubiquitinationin in the cell. Together our data support a model in which Alix recruits Nedd4-1 to facilitate HIV-1 release mediated through the LYPXnL/Alix budding pathway via a mechanism that involves Alix ubiquitination.

STRUCTURE OF THE HIV-1 CA PENTAMER

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The mature capsids of HIV-1 and other retroviruses are fullerene protein shells that package the viral RNA for delivery into new host cells and facilitate proper reverse transcription of the genome. Retroviral capsids are composed of around 250 hexamers and exactly 12 pentamers of the viral CA protein, which are arranged on a variably curved hexagonal lattice. The lack of strict global symmetry in retroviral capsids has precluded traditional high-resolution structural analyses. Studies of these capsids have therefore employed symmetric model systems, derived from pure recombinant proteins in vitro.

We have previously used a disulfide crosslinking strategy to facilitate the purification and crystallization of soluble HIV-1 CA hexamers. The resulting structures revealed in atomic detail the quaternary interactions that stabilize the hexamer, and the structural variations required to form a variably curved hexagonal lattice. We have recently used a similar strategy to determine X-ray crystal structures of the HIV-1 CA pentamer at 2.5 Å resolution. The new structures reveal that indeed, the CA pentamer and hexamer are quasi-equivalent. The same protein surfaces are used to form both oligomers, with the N-terminal domain of CA forming hexameric or pentameric rings, and the C-terminal domain forming a mobile belt around the central ring. Pentamer formation appears controlled by electrostatics, and may involve a balancing of attractive and repulsive ionic interactions between the N-terminal domains.

SERINE RESIDUES IN THE P6 PROTEIN OF HIV-1 ARE PHOSPHORYLATED BY PKC AND REGULATE VIRAL CORE ASSEMBLY

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The p6 protein of HIV-1 Gag contains the two late assembly (L-) domains that recruit certain host cell factors in order to facilitate the release of nascent virions from infected cells. In addition, HIV-1 p6 has been characterized as the major phosphoprotein in virus particles. It was further observed that p6 can be modified at Thr, Ser, and, to a smaller extent, at Tyr residues indicating that p6 should be a substrate for several kinases. Originally, the mitogen-activated protein (MAP)-kinase ERK-2 was identified to mediate the phosphorylation of p6 in position Thr-23, a modification that was suggested to regulate the assembly and release of progeny virions.
In the present study, we identified p6 as a substrate of the protein kinase C (PKC) that phosphorylates Ser residues in positions 14, 25, and 40. Individual substitution of all Ser residues in p6 revealed that only Ser-40 is important for virus replication in T-cell lines and in human lymphocyte tissue cultivated *ex vivo*, while combined mutation of all three PKC-sites completely abrogated replication. Most intriguingly, mutation of the PKC sites had no influence on L-domain mediated virus release. In contrast, the specific infectivity of the virions was reduced when Ser-40 was exchanged and completely lost when all PKC sites were mutated. Furthermore, it was observed that the integrity of the PKC sites is crucial for the final processing of CA from p25 to p24. Electron microscopy revealed that this defect in CA processing in turn leads to an irregular morphology of the virus core and the formation of an electron dense extra core structure. Thus, the cumulative results support a novel function of p6 that does not affect virus release but stimulates maturation of CA in a process that might be governed by PKC phosphorylation.

RESCUE OF HIV-1 BUDDING DEFECTS BY TARGETING UBIQUITIN LIGASE HECT DOMAINS TO GAG

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PPxY-type L domains are thought to promote retroviral budding through the recruitment of Nedd4 family ubiquitin ligases. Surprisingly, the budding and infectivity of HIV-1 L domain mutants is potently enhanced by ectopic Nedd4-2s, even though HIV-1 Gag lacks PPxY motifs. Nedd4-2s is a Nedd4-like ubiquitin ligase that associates with HIV-1 Gag via a naturally truncated C2 domain. In addition, Nedd4-2s harbors four ligand-binding WW domains and a catalytic HECT domain. We now find that the truncated C2 domain of Nedd4-2s can be functionally replaced by cyclophilin A (CyPA), which binds to the CA domain of HIV-1 Gag. Furthermore, when targeted to Gag through CyPA, or alternatively through the truncated Nedd4-2s C2 domain, the HECT domains of most but not all members of the Nedd4 ubiquitin ligase family were sufficient to efficiently rescue HIV-1 budding. Notably, chimeras based on ubiquitin ligases with exquisite specificity for the synthesis of K63 ubiquitin chains were active, indicating that the synthesis of K48 chains is not required to function in viral budding. Interestingly, certain HECT domain fusion proteins and intra-HECT domain chimeras induced comparable levels and patterns of HIV-1 Gag ubiquitination, even though they differed dramatically in their ability to rescue HIV-1 budding. Thus, our results strongly argue against the possibility that Gag is the functional target of HECT domain-induced ubiquitination.

STEPS IN HIV-1 MATURATION STUDIED WITH CRYO ELECTRON TOMOGRAPHY

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HIV-1 buds out from the infected cell in an immature, non-infectious form. Immature virions contain a partial spherical shell with Gag organized as a hexameric lattice. This lattice is well ordered at the CA level, and is linked via rod-like densities to an inner, disordered layer that corresponds to NC-RNA [1]. Maturation requires proteolytic cleavage of the Gag polyprotein at five positions, leading to a dramatic change in the organization of the CA domain and formation of the conical core. To shed light on the maturation process we analyzed viral variants containing point mutations abolishing cleavage at individual or multiple Gag cleavage sites [2]. For each variant we used cryo-electron tomography and subsequent subtomogram averaging to resolve the structure of the Gag lattice.

Mutation of all proteolytic sites in Gag gave rise to particles that were structurally indistinguishable from immature virions due to a non-functional or inactivated protease. If only a single cleavage site between MA and CA was retained, there was also no change in the structure of the Gag lattice, but the distance between the CA layer and the membrane became more variable. If all cleavage sites downstream of SP1 (NC-SP2-p6) could be processed leaving an uncleaved MA-CA-SP1 product, the CA lattice and rod like densities were maintained, while the NC-RNA complex condensed in the particle centre. If only the cleavage site between MA and CA was mutated, processing at the wild-type downstream site between CA and SP1 was also reduced. Despite partial cleavage at this junction, the hexagonal immature lattice and the rod-like structures were retained. Mutating only the cleavage site between CA and SP1 produced virions with a condensed NC-RNA complex that contained no visible immature Gag lattice.

These results indicate that disassembly of the immature Gag lattice requires cleavage both upstream and downstream of CA. Furthermore, the rod-like densities protruding below the CA layer maintain their structure even if the NC-RNA complex has condensed, and the CA-SP1 junction has been partially cleaved, suggesting that they represent a stable assembled oligomeric structure.

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A ROLE FOR CLATHRIN IN RETROVIRUS PARTICLE MORPHOGENESIS

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To establish and maintain productive infection in host species, retroviruses employ numerous host factors for replication. We used a mass spectroscopy approach and highly purified HIV-1 virus-like particles to identify host proteins that might be involved in particle morphogenesis.

We identified clathrin as a component of highly purified protease-defective HIV-1 virus-like particles (VLPs). Notably, clathrin was exceptionally abundant in these particles, and was present at almost the same levels as the Pol protein. Moreover, clathrin incorporation was highly specific – it was completely absent from particle generated using the HIV-1 Gag protein alone. This specificity of incorporation was further confirmed by the fact that clathrin incorporation was almost undetectable in particles generated by GagPol proteins bearing point mutations in the RT or IN domains of Pol. Remarkably, several other retroviruses (SIVmac, MLV and M-PMV) also packaged clathrin into virions. However in each of these cases, Gag rather than Pol was responsible for recruiting Gag into virions. Additionally, for all three retroviruses we could identify specific peptide sequences encoded within the Gag protein that mimicked sequences found in cellular clathrin adapter proteins and were entirely responsible for clathrin incorporation. Correspondingly, the amino-terminal beta-propeller domain of the clathrin heavy chain (HC) that is normally responsible for binding to clathrin adapters was sufficient for clathrin incorporation into VLPs.

Mutations in viral proteins that blocked clathrin incorporation, or perturbation of cellular clathrin had several effects on virion morphogenesis that differed according to which particular virus or viral protein was analyzed. Deleterious effects on viral protein stability, the assembly and release of virions and/or virion infectiousness could be induced by perturbing clathrin recruitment. Collectively, these data suggest an important role for clathrin-Gag and/or clathrin-Pol interactions in the genesis of retroviral particles.

IDENTIFICATION OF HIV-1 CAPSID ASSEMBLY INHIBITORS

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HIV capsid assembly is a late essential step in the retroviral life cycle and is not targeted by current antiretrovirals. We employed an in vitro translation system, which faithfully models HIV assembly in living cells, to screen a small molecule library for HIV-1 capsid assembly inhibitors. Several hits were obtained in this screen. Here, we report the antiviral activity of selected compounds and provide insights into their mechanism of action. Several compounds found to be active in the cell free system also suppressed HIV-1 replication in a T cell line, indicating that the experimental system is suitable to identify antivirals. Six compounds, representing 5 chemical scaffolds, with CC_{50} and IC_{50} values in the lower micromolar range, were selected for further characterization. All of the selected compounds showed pan-lentiviral activity with similar IC_{50} values for HIV-1, HIV-2 and SIV, but inhibited different stages of the viral life cycle. Compound 4.1 showed a strong potential to inactivate cell-free virus which might account for its early effect on HIV-1 infection. It could be shown that compound 4.1 does not interfere with virus release from chronically infected Jurkat cells, but diminishes the infectivity of released virions. A FACS-based FRET assay revealed that compound 4.1 disturbed Gag-Gag interactions, and Gag processing intermediates, which were recently shown to inhibit viral infectivity, were detected in virion preparations treated with compound 4.1. In agreement with these observations, a preliminary electron microscopy analysis suggested that treatment of infected cells leads to release of virions with aberrantly formed capsids. Collectively, our results indicate that compound 4.1 might interfere with appropriate Gag folding, and might thereby inhibit formation of infectious particles.

RAB11A MODULATES HIV-1 ENV INCORPORATION

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HIV-1 is an enveloped virus and the incorporation of Env is crucial for the viral infectivity. Although under extensive study for decades, the cellular locations and molecules involved in Env incorporation have not been clearly defined. We propose that intracellular trafficking of Env through recycling pathways is an essential determinant of the specific virion incorporation of Env. To test this hypothesis, we expressed dominant-negative and constitutively-active forms of Rab11a, and monitored Env incorporation at the budding site and on released viral particles. Dominant-negative Rab11a did not alter Env incorporation, which seemed to contradict our hypothesis. Surprisingly, however, we found that expression of a constitutively active form of Rab11a reduced Env incorporation dramatically. Particle infectivity was correspondingly decreased by this intervention. Next, we examined the effect of constitutively-active Rab11a on Env lacking the long cytoplasmic tail. Constitutively-active Rab11a had no effect on incorporation on NL4.3 del144 Env incorporation, indicating that the specific incorporation defect could be mapped to the Env cytoplasmic tail. EM analysis of particles released in the presence of constitutively active Rab11a revealed markedly-diminished Env incorporation. We conclude that altered recycling or rapid recycling from the pericentriolar recycling compartment prevents Env from reaching the particle assembly site and shunts it toward alternative intracellular sites.

LIPID DEPLETION MODULATES TRIM5ALPHA-MEDIATED RETROVIRAL RESTRICTION

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The cross-species restriction factor TRIM5α is a member of the tripartite motif (TRIM) family of proteins and affects the retroviral life cycle. Human TRIM5α (TRIM5αhu) potently restricts N-MLV infection, but not N/B-MLV infection, while rhesus monkey (TRIM5αrh) blocks both early and late phases of the HIV-1 life cycle, but does not block SIV infection or production. Detailed mechanisms involved in the TRIM5α-mediated retroviral restriction still remain elusive. To better understand the mechanisms of TRIM5α mediated antiviral activities, we first examined a cellular binding partner of TRIM5αrh. Immunoprecipitation of HA-tagged TRIM5αrh and subsequent MS analysis identified the membrane raft-associated protein flotillin-1/reggie-2 as a potential TRIM5αrh interaction partner. Immunohistochemistry studies confirmed the co-localization of TRIM5αrh cytoplasmic bodies with flotillin-1/reggie-2, as well as another lipid raft marker caveolin-1. A detergent-free protein flotation assay on HA-tagged TRIM5α identified TRIM5α in flotation fractions alongside lipid microdomain-associated proteins, including flotillin-1/reggie-2. Moreover, lipid depletion disrupted prominent TRIM5αrh cytoplasmic body formation. These observations suggest the association of TRIM5αrh with subcellular lipid microdomains, and a possible role of flotillin-1/reggie-2 or cellular lipids in TRIM5α-mediated antiviral activities. Next, we examined the influence of flotillin-1/reggie-2 knockdown and cellular lipid depletion on TRIM5α-mediated retroviral restriction. Although knockdown of flotillin-1/reggie-2 expression in rhesus monkey FrhK4 cells showed no significant effects on TRIM5αrh-mediated early and late restriction of HIV-1 replication, depletion of cellular lipids impaired the TRIM5αrh-mediated late restriction of HIV-1, but not SIVMAC. Moreover, lipid depletion prior to infection strongly increased infectivity of N-MLV, but not N/B-MLV, in human TE671 cells, suggesting that lipid depletion impairs TRIM5αhu-mediated N-MLV restriction. Importantly, lipid repletion prior to infection partially restored the TRIM5αhu-mediated N-MLV restriction. Our data therefore demonstrated the critical role of cellular lipids for efficient TRIM5α-mediated antiviral activities.

THE LOCALIZATION OF APOBEC3H HAPLOTYPES IN HIV-1 VIRIONS DETERMINES ITS ANTIVIRAL ACTIVITY

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Several single nucleotide polymorphisms (SNPs) have been mapped to the coding regions of each of the seven human APOBEC3 (A3) but their functional impact remains to be explored. Recently, we and others have found that the single domain cytidine deaminase A3H is polymorphic in sequence. One of the haplotypes (A3H HapII-RDD) displays a strong antiviral activity. The goal of this study was to elucidate the mechanisms underlying the differences in activity between A3H haplotypes.
In addition to the naturally occurring A3H HapI-GKE and HapII-RDD proteins (which differ at three amino acid positions) we constructed a panel of 6 site-directed mutants that contain all possible combinations of the mutations. A series of transfections, infections and GST pull-down experiments was performed to assess A3H protein stability, the requirements for A3H-Gag interactions and A3H virion incorporation. The catalytic activity of each A3H variant was assessed using an E. coli mutator assay.
A3H HapII-RDD restricts HIV-1 NL4-3 ~10-fold more efficiently than HapI-GKE. Since this difference in antiviral activity strongly correlates with protein expression levels in the producer cell we anticipated to observe differences in the amount of A3H being packaged. Contrary to our expectations, incorporation of A3H into the HIV-1 virions was equally efficient for all the variants. When tested in a bacterial mutator assay, both A3H haplotypes showed similar deaminase activity upon normalization for differences in expression. GST pull-downs and virion incorporation experiments using Gag deletion mutants demonstrated that each A3H haplotype interacts with a different Gag region; A3H HapII-RDD associates with nucleocapsid (NC) in an RNA dependent manner whereas A3H HapI-GKE interacts exclusively with capsid.
A3H is the first human cytidine deaminase for which haplotypes with distinct antiviral activity have been described. Our results suggest that the single deaminase domain of A3H is both catalytically active and functions in RNA binding/virion incorporation. However, the interaction of A3H HapI-GKE with Gag capsid but not NC likely prevents its access to the reverse transcription complex and thereby limits its antiviral activity.

GAG RNA AND MEMBRANE BINDING DETERMINANTS FUNCTION COORDINATELY DURING HIV-1 GAG PARTICLE ASSEMBLY

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Prior assembly studies suggest that three interactions drive the assembly of HIV-1 Gag into particles: Gag-binding to an RNA scaffold that promotes Gag multimerization, Gag multimers binding to the plasma membrane that form larger complexes, and Gag-Gag interactions which condense these loose structures into a tightly knit particle shell. Despite much progress in identifying these processes, it is not clear whether they act discretely or cooperatively. Previously, we found that Gag required one of two RNA binding sites, either NC or a basic region of MA within Gag for efficient particle formation (Ott et al., J. Virol. 79:13839), supporting the RNA scaffold model. A follow-up study of the kinetics of wild-type and NC mutant Gag particle production indicated that NC-mediated binding of RNA is an initiating event in Gag assembly (Ott et al., J. Virol. 83:7718), confirming a prediction of the RNA scaffold model. To examine the interdependence of RNA binding and plasma membrane binding during Gag assembly, we co-expressed our MXNX proviral mutant construct, which contains a mutated MA RNA-binding site and a nearly complete NC deletion, with a myristylation mutant (myr-). Neither of these mutants efficiently produces particles by themselves, yet particle incorporation for each is readily complemented by wild-type Gag. Co-transfection experiments between these two constructs revealed that MXNX and myr- could not rescue each other, indicating that RNA binding and plasma membrane binding are mutually dependent. In contrast each mutant alone, carrying either the MA mutation (MX) or the NC deletion (NX) could rescue myr- Gag. These data indicate that RNA binding and membrane binding are coordinated events that function together during HIV-1 Gag assembly.

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ADAPTATION OF HIV-1 TO ESCAPE RHESUS MONKEY TRIM5-ALPHA.

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In Old World monkeys, HIV-1 encounters blocks mediated by the restriction factors TRIM5α, APOBEC3G and tetherin. Overcoming these blocks may allow the development of HIV-1-like viruses that infect macaques. In the present work, we used a protocol previously employed to adapt HIV-1 to replicate in CD4-negative cells [1] and in cells expressing common marmoset CD4 and CXCR4 [2]. We successfully adapted an HIV-1 isolate to replicate in HeLa-CD4 cells expressing rhesus monkey TRIM5α (rhTRIM5α). A single residue change that arose during the first passage of the virus, and is located in the base of the CypA-binding loop of the capsid protein, was sufficient to allow the virus to replicate in HeLa-CD4 cells stably expressing rhTRIM5α. Competition assays with virus-like particles and assays directly measuring rhTRIM5α binding to CA-NC tubes demonstrate that the mechanism of escape of this capsid mutant involves a reduced binding affinity for rhTRIM5α. Cyclophilin A binding to the adapted virus capsid protein was comparable to that of the wild-type capsid. The combination of this capsid mutant with other changes designed to overcome restriction might help to generate a more HIV-1-like virus able to replicate in macaques.

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MUTATIONAL ANALYSIS OF THE DETERMINANTS FOR CYTOPLASMIC LOCALIZATION AND ANTI-RETROVIRAL ACTIVITY OF APOBEC3B.

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Human APOBEC3B (A3B) has been described as a potent inhibitor of retroviral infection and retrotransposition. However, we found that the predominantly nuclear A3B did not restrict infection by HIV or HIVDvif, while significantly inhibiting LINE-1 retrotransposition. A chimeric mutant A3G/B, in which the first 60 amino acids of A3B were replaced with those of A3G, was able to restrict HIV, HIVDvif and HTLV-1 infection, as well as LINE-1 retrotransposition. In contrast to the exclusively cytoplasmic A3G, which is inactive against LINE-1 retrotransposition, A3G/B protein, while mainly localized to the cytoplasm is also present in the nucleus. Further mutagenesis analyses revealed that residues 18, 19, 22 and 24 in A3B are the major determinants for nuclear vs cytoplasmic localization and anti-retroviral activity. HIVDvif packages A3G, A3B and A3G/B with close to equal efficiencies into particles. As in A3B, mutations E68Q and E255Q in the active centers of A3G/B resulted in loss of the inhibitory activity against HIVDvif while retaining activity against LINE-1 retrotransposition. The low inhibition of HIVΔ vif by A3B correlates with a low mutation rate in viruses transmitted in the presence of A3B. In contrast, viruses, which had been exposed to A3G/B, showed a high number of G to A transitions. The mutation pattern was similar to that previously reported for A3B with a preference of the TC context (84%) followed by CC (16 %). In summary, these observations suggest that the nuclear localization of A3B targets it to inhibit transposition of LINE-1 and LINE-1 dependent retro-elements, and that redirecting it to the cytoplasm enables it to restrict retroviruses.

MLV DIVERSITY IN WILD MOUSE SPECIES AND THE ROLE OF APOBEC3

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Infection of the germ line by retroviruses has led to endogenous proviruses, most of which have become inactivated due to mutations. Nonecotropic murine leukemia viruses (MLVs) are endogenous gammaretroviruses and can be subdivided into three groups: xenotropic (XMV), polytropic (PMV), and modified polytropic (MPMV). Previous work in our laboratory has identified 49 nonecotropic MLV proviruses in the sequenced genome of C57BL/6J inbred mice. This work also found evidence for editing by APOBEC3 (mA3), a host defense mechanism that caused G-to-A mutations during viral DNA synthesis at the time of integration of PMV and MPMV, but not in XMV proviruses. Since inbred mice were derived from crosses of several different wild subspecies of mice (*Mus musculus domesticus* and *M. m. molossinus*, itself a natural hybrid between *M. m. musculus* and *M. m. castaneus*), which may differ in A3 activity, we sought to identify and characterize endogenous MLV proviruses and their integration sites in wild mouse species.

We used PCR primers to amplify fragments representing specific proviruses, solo LTRs, and unoccupied integration sites identified in the C57BL/6J mouse genome. Only 7 proviruses were found at the same location in wild mouse species, indicating that integration occurred relatively recently in the lineage or lineages that gave rise to inbred lines. No such proviruses were found in *M. m. domesticus*, but we identified 7 XMV and MPMV proviruses in *M. m. molossinus*, *M. m. musculus*, and *M. m. castaneus* integrated in the same locations in the genomes as in the inbred strain. The presence of proviruses varies among individuals of *M. m. molossinus*, indicating that they integrated after the origin of this subspecies and are not yet fixed in the population sampled. The 5' and 3' LTRs of these identified proviruses were identical in sequence to one another and to the corresponding LTRs in the B6 genome, indicating that these integrations occurred quite recently. We are currently identifying and sequencing more proviruses in the genomes of both *M. m. molossinus* and other wild mouse species using primers targeting provirus-specific regions and B1 repetitive DNA elements. We are also examining the *APOBEC3* gene in the various species to explore the possibility that APOBEC3 function may differ in certain wild mice species and to see if differences correlate with the proviruses present in their genomes.

MECHANISM OF HIV-1 NEF INDUCED INTRACELLULAR LCK ACCUMULATION

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The Nef protein is a critical factor for AIDS pathogenesis that substantially increases HIV replication in vivo via the modulation of multiple host cell transport and signal transduction processes. In T lymphocytes, Nef is well known to affect actin remodeling and chemotaxis as well as to modulate cell surface exposure of transmembrane receptors. In addition, Nef also causes pronounced intracellular accumulation of the Src kinase Lck that is only peripherally associated with the inner plasma membrane leaflet; the underlying mechanism and functional consequences of this highly conserved Nef activity are currently unclear.
Using spinning disc confocal and immuno-electron microscopy we identify the intracellular compartment in which Lck accumulates in the presence of HIV-1 Nef as the Trans Golgi network. Real time imaging reveals that Nef potently inhibits the otherwise highly dynamic intracellular transport of Lck from intracellular membranes to the plasma membrane. While this effect relies on the SH3 domain binding motif in Nef and acylation of Lck, known protein interaction motifs of the kinase and colocalization with Nef are dispensable. Consistent with a more general effect on membrane trafficking, Nef also induces intracellular accumulation of other Src kinase family members. Kinetic analysis of Lck transport demonstrates that Nef selectively acts on newly synthesized Lck molecules to prevent their initial targeting to the plasma membrane. Functional studies indicate that this block in plasma membrane translocation of Lck is the mechanism by which Nef in T lymphocytes prevents recruitment of Lck to contact sites with antigen presenting cells. Together, these findings unravel a novel mechanism by which Nef affects plasma membrane targeting of membrane-associated host cell factors to optimize the environment in HIV-1 infected T lymphocytes.

ANALYSIS OF TETHERIN ANTAGONISM BY HIV-1 M AND O GROUP VPU PROTEINS

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Zoonosis of SIVcpz has given rise to both pandemic (M) and non-pandemic (O and N) groups of HIV-1, as well as SIV infection of Gorillas (SIVgor). SIVgor itself may also have transmitted to humans giving rise to HIV-1 group P. These lentiviruses encode accessory proteins, including Vpu, which has two important roles. Vpu removes CD4 protein from the cell membrane to prevent super-infection and aid virus release. Vpu from M group HIV-1 also improves virus release by antagonizing tetherin (CD317, BST2) which acts to tether newly formed virions to the infected cell surface. Here we show that Vpus from O group HIV-1 Ca9, BCF06 and BCF07 are not able to counteract human tetherin, although they are still able to remove cell-surface CD4. We hypothesize that an inability to antagonize human tetherin has contributed to O group viruses failing to transmit in humans to pandemic levels. Characterization of chimeric and mutant O groups Ca9 and BCF06, and M group Vpu domains demonstrated that the Vpu/tetherin interaction is complex and involves several domains. We have also identified specific residues within the Beta-TrCP recognition signal that are important for tetherin antagonism, as well as specific residues within the transmembrane domain that have been under positive selection. The specific localization of the various Vpus has also been studied. This work aims to shed light on the molecular details of how Vpu acquired anti-tetherin activity and led to a pandemic HIV infection of humans.

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CHARACTERISATION AND FUNCTIONAL IMPLICATIONS OF THE INTERACTION BETWEEN THE APOBEC3 AND ARGONAUTE PROTEIN FAMILIES.

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The APOPEC3 family of cytidine deaminases (A3A-A3H) play an important role in host mediated antiviral defence against retroviruses and retroelements, including HIV-1. Although much has been discerned about the APOBEC3 proteins in terms of mechanisms of antiviral resistance, the cellular function of these proteins remains poorly defined. In an attempt to address this and further elucidate their anti-viral activity, several cellular proteins that interact with A3G have been identified. The most intriguing of these are the Argonaute protein family as they interact with A3G in a manner which is at least partially resistant to RNase treatment. The Argonautes are integral components of RISC, which is involved in miRNA mediated translational repression and mRNA decay. Components of this pathway, as well as A3G and silenced mRNAs have been shown to localise to discrete cytoplamic foci termed Processing (P) Bodies. We aim to better understand the extent of the associations between these two protein families and the relevance this may have to APOBEC3 cellular and/or anti-viral activities.
We have carried out a detailed analysis of APOBEC3 family members in terms of interaction with the Argonautes and co-localisation to P-bodies. Our findings show that the most potent antiviral proteins, A3F, A3G and A3H are able to interact with several of the Argonaute proteins, while no detectable interaction was found with the rest of the APOBEC3 family. With regards to P-body localisation, once again, A3F, A3G and A3H all showed strong co-localisation with Ago2 as did A3D/E, which has a more modest anti-viral effect. The remaining APOBEC3 proteins displayed weak or no co-localisation. Therefore there appears to exist a correlation between the anti-viral activity of APOBEC3 family members and interactions with the Argonaute proteins. This specificity has led us to further investigate the functional consequences of this interaction. It has previously been reported that members of the APOBEC3 family are able to counteract the repression mediated by miRNAs implicating a role for these proteins in the regulatory control of cellular mRNA translation. We are attempting to extend these findings through the utilisation of miRNA based reporter assays and aim to further investigate any correlates which may be present between this potential cellular function of the APOBEC3 proteins, association with the Argonautes, localisation to P-bodies and anti-viral phenotypes.

FUNCTIONAL CHARACTERIZATION OF THE NEF-LIKE ACTIVITY OF GAMMARETROVIRAL GLYCOSYLATED GAG ON VIRION INFECTIVITY

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Most gammaretroviruses encode a glycosylated transmembrane Gag molecule (glycogag) translated from a CUG initiation codon upstream and in-frame with gag. We have recently established that glycogag derived from murine leukemia virus is an infectivity factor able to fully rescue the defective infectivity of Nef-negative HIV-1. Nef and glycosylated Gag share major functional features. The activity of the two proteins similarly depends on the producer cell-type and the nature of the envelope glycoprotein. In addition, Nef and glycogag co-localize in intracellular, perinuclear compartments.

We have now further characterized the functional similarity between the two retroviral factors. Nef and glycogag affect an equally early step of the retroviral life cycle. In addition, the activity of glycogag on infectivity is inhibited by agents which impair also the function of Nef. Altogether, our data strongly suggest that the two retroviral factors represent a case of convergent evolution.

We have established that the activity on retrovirus infectivity resides within the intracellular portion of glycogag. We have evidence that only the transmembrane-proximal region is required for this function. Intriguingly, comparative analyses of glycogag sequences derived from different gamma-retroviruses shows that this region is highly divergent and the least conserved within glycogag. We are now testing the ability of different glycogag alleles to replace the activity of Nef on HIV-1 infectivity. This will establish whether the activity on infectivity is conserved across glycogag-encoding gammaretroviruses and will identify molecular determinants crucial for this function.

GENERATION OF RESTRICTION FACTOR-TRANSGENIC DOMESTIC CATS.

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Species-specific restriction factors (RFs) may have therapeutic potential. FIV is restricted by several primate TRIM5α & TRIMCyp proteins, unusually including both aotus and macaca genus TRIMCyps, and there are no feline orthologs. If transgenesis with RF genes were feasible, domestic cat models could have potential for a number of goals, including testing hypotheses that introducing a RF can render a species genetically immune to a lentivirus, reduce in vivo replication or prevent AIDS.
We have now achieved transgenesis in the domestic cat, using single cell embryo microinjection of lentiviral vectors that co-encode a RF & GFP. We first compared FIV restriction of primate TRIM5α & TRIMCyp alleles in cultured feline cells. M. mulatta TRIMCyp (rhTC) was potent, reducing FIV infectivity > 3 logs. Vector and feline-specific reproductive biotechnology parameters were optimized iteratively to enable transgenesis. Microinjecting before or after fertilization had no effect on embryo development or transgene expression. Pre-fertilization injection produced less mosaicism. Expressing GFP + rhTC (rhTC.GFP vector) or GFP alone showed that adding rhTC had no deleterious effects on feline blastocyst development.
Transfer of rhTC.GFP vector-microinjected embryos into reproductive tracts of 6 queens using empirically optimized queen pre-conditioning and embryo transfer protocols resulted in two live, healthy male transgenic (TG) kittens. Southerns & PCR on tail & PBMC DNA demonstrated each cat has several insertions. TG but not control PBMCs were GFP-fluorescent. WB revealed GFP and rhTC in TG but not control PBMCs. 2 other pregnancies resulted in premature birth of 6 fetuses, all TG, and a full-term, morphologically normal TG kitten that died of obstetrical complications. Autopsies showed transgene expression in multiple organs. We conclude that feline-optimized lentiviral transgenesis methods can generate multi-gene TG domestic cats. RF-TG cats are being bred, sperm-banked, and challenged with animal-titered FIV. Further transfers are in progress with different RFs, promoters. The approach will aid analysis of RF biology in vivo and RF-based gene therapy. In addition to potential for conferring immunity against this lentivirus, transgenesis in this accessible species may foster modeling of other diseases.

NON-PRODUCTIVE REPLICATION OF HIV-1 IN RENAL PODOCYTES: IMPLICATIONS FOR HIVAN PATHOGENESIS.

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HIV-1 associated nephropathy (HIVAN) is a renal disease almost entirely limited to individuals of African descent. Genome–wide association studies have recently identified MYH9 as a candidate gene associated with the high risk for development of HIVAN in African Americans. The cellular target involved in the development of HIVAN is the renal epithelium, most notably podocytes and tubular cells. Since podocytes are the critical components of the blood filtration barrier, loss of podocytes into urine as a result of HIV-1 infection may promote glomerular diseases including HIVAN. However, the mechanism of podocyte infection in HIVAN is controversial. One of the current hypotheses is that productive infection of podocytes by HIV-1 and expression of HIV-1 accessory genes causes the cellular injury. However, prior our study, this hypothesis was never tested in vitro using podocytes and wild-type HIV-1. The reported lack of classical HIV-1 entry receptors CD4, CXCR4, or CCR5 in podocytes suggests that virus entry through this pathway is unlikely. We have shown that transfection of podocytes with CD4 and CXCR4 expression vectors restored productive replication of X4 HIV-1 to the levels observed with VSV-G pseudotyped HIV-1, confirming that lack of entry receptors is a major barrier to establishing productive infection in podocytes. Further, we have shown that wild-type HIV-1 readily enters podocytes through a dynamin-dependent endocytosis and partially colocalizes with recycling endosomes and late endosomes/lysosomes. However, the majority of the internalized virus escapes degradation and is released back to the culture medium as a fully infectious virus. We have detected only a limited and transient accumulation of HIV-1 reverse transcription DNA products, suggesting that HIV-1 is unable to establish productive infection in podocytes. This was further confirmed by detection of HIV-1 Gag and unspliced HIV-1 RNA only very early in infection, indicating they originate from virion-associated endocytosed HIV-1 particles rather than de novo synthesis. We conclude that the reported in vivo detection of HIV-1 nucleic acids and proteins in glomerular podocytes from HIVAN individuals may reflect endocytosed virions rather than productively replicating virus. A new mechanism describing an abortive HIV-1 infection leading to podocyte injury in HIVAN will be discussed.

EVOLVED CONFORMATIONAL ADAPTATIONS DIRECT ASIAN MACAQUE TRIMCYPS TO DIFFERENT LENTIVIRAL LINEAGES

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TRIMCyps are anti-retroviral proteins that have arisen independently in New World and Old World primates. All TRIMCyps comprise a CypA domain fused to the tripartite domains of TRIM5α but they have distinct lentiviral specificities, conferring HIV-1 restriction in New World owl monkeys and HIV-2 restriction in Old World rhesus macaques. Here we provide evidence that Asian macaque TRIMCyps have switched restriction specificity between different lentiviral lineages during their evolution, resulting in species-specific alleles that target different viruses. Structural, thermodynamic and viral restriction analysis suggests that a single mutation in the Cyp domain, R69H, occurred early in macaque TRIMCyp evolution, expanding restriction specificity to the lentiviral lineages, found in African green monkeys, sooty mangabeys and chimpanzees. Subsequent mutations in different species have enhanced restriction to particular viruses but at the cost of broad specificity. We reveal how specificity is altered by distant scaffold mutations that modify surface electrostatics and propagate conformational changes into the active site. Our results suggest that lentiviruses may have been important pathogens in Asian macaques despite the fact that there are no reported lentiviral infections in macaque populations.

IMMUNE RESPONSES IN XMRV-INFECTED RHESUS MACAQUES: SEROLOGICAL MARKERS OF XMRV INFECTION

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Background: Xenotropic Murine Leukemia Virus-related Retrovirus (XMRV) is a human gammaretrovirus recently identified in prostate cancer tissue and in lymphocytes of patients with chronic fatigue syndrome. To establish the etiologic role of XMRV infection in human disease requires large scale epidemiological studies; development of serological assays to detect XMRV-specific antibodies (serological markers) would greatly facilitate such studies. An understanding of the dynamics of the antibody response elicited by infection with XMRV is essential for development of reliable serological assay(s).
Methods: Three rhesus macaques (2 males, 1 female) were inoculated intravenously with XMRV; two were re-inoculated on day 158. Blood was collected throughout the course of infection. XMRV-specific antibody responses were monitored by Western Blot (WB) using XMRV lysate and recombinant viral proteins. In addition, prototype serologic assays to detect antibodies to *env* gp70, *env* p15E and *gag* p30 were developed using recombinant antigens on the high-throughput automated ARCHITECT instrument system (Abbott Diagnostics).
Results: By WB analysis, all three macaques developed antibody responses to *env* and *gag* proteins during the second week of infection. Specific antibody responses to *env* gp70 and *env* p15E were detected at 9 days post infection; antibodies to *gag* p30 were detected by days 11-14. All three antibody responses persisted to 158 days and were substantially boosted by re-inoculation. Two macaques exhibited weak and transient antibody responses to *gag* p15 and *gag* p10. Based on WB analysis, antibody responses to *env* gp70, *env* p15E and *gag* p30 were identified as useful markers for serological assay development. The prototype ARCHITECT assays demonstrated 100% sensitivity by detecting all WB positive serial bleeds from the XMRV-infected macaques.
Conclusions: This study characterized the dynamic process of antibody development in XMRV-infected macaques and identified serological markers useful for detection of XMRV infection. The prototype XMRV-antibody assays will facilitate large-scale epidemiological studies.

ANTI-HIV-1 NUCLEOCAPSID RNA APTAMERS REVEAL A POSSIBLE CONSENSUS RNA MOTIF IN THE PACKAGING SIGNALS OF DIVERSE RETROVIRUSES

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Genome encapsidation by HIV-1 involves specific recognition and subsequent packaging of two copies of the full-length viral genomic RNA in the cytoplasm of infected cells. This specific packaging is facilitated by an RNA sequence (known as ψ) in the 5'-UTR of the virus which is recognized by the nucleocapsid (NC)-domain in the Gag polyprotein. The interaction of the ψ− sequence of a retrovirus with its cognate NC protein is believed to be highly specific and cross-packaging between retroviruses is known to occur but at a low efficiency.

We raised RNA aptamers against the HIV-1 Gag polyprotein and identified two aptamers that bound specifically to DP6-Gag (a version of HIV-1 Gag lacking p6) and the NCp7 protein. The two aptamers specifically bound to HIV-1 but not to SIV_{mac} or RSV- Gag. In order to understand the nature of the interaction of the NC-specific aptamers with HIV-1 NC, we designed an *in vitro* competition assay where purified NC protein was incubated with a mixture containing radiolabeled aptamer RNA and increasing concentrations of unlabeled RNA transcripts encoding the ψ− RNAs from several retroviruses. While a robust competition for binding to NC was observed when ψ− RNA from HIV-1 was used as a competitor, control RNAs like yeast tRNA, an MA-specific RNA aptamer or a ψ− RNA from RSV, an alpharetrovirus, were unable to compete for binding. Interestingly, HIV-2, SIV and MuLV ψ− RNAs also strongly competed with the aptamers for binding to HIV-1 NC similar to that of HIV-1 ψ−RNA. While HIV-1 NC has been shown to recognize the ψ−sequences from HIV-2 and SIV_{mac}, it is not known to interact with the ψ−sequence of MuLV. Similar results were also obtained when HIV-1 NC in the binding assays was replaced with DP6-Gag. Our results suggest a novel possibility that the ψ− regions of diverse retroviruses contain a conserved RNA-motif and discrimination between the genomic RNAs by HIV-1 NC might require a second signal unique to the virus. The results of our ongoing experiments to delineate the minimal binding domains of various retroviral ψ sequences necessary for this competitive binding will be presented.

To determine if the aptamers could function as molecular mimics *in vivo*, we replaced the ψ-region (SL1- or SL3- stem loops) of pNL4-3.Luc.R-E- with the NC-specific aptamers. While the replacement of the ψ−sequence with the aptamer sequence did not inhibit viral protein processing or assembly, we observed that all of the viruses had reduced infectivity (3-10 fold) compared to wild-type viruses. We are currently examining the levels of genomic RNA encapsidated into these virions by quantitative RT-PCR to study if the aptamers could in fact mimic the ψ-region of HIV-1 *in vivo*.

THE IMPACT OF HIV-1 CAPSID MUTANTS ON INFECTION OF PRIMARY HUMAN MACROPHAGES

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Lentiviruses including HIV-1 can efficiently infect non-dividing cells, whereas gamma retroviruses require cell division. Though the details remains unclear this property is thought to be due an ability of lentiviruses to recruit host nuclear import proteins and traverse the nuclear pore. Here, we show that mutations in HIV-1 capsid (CA) that impact on the ability to infect non-dividing cells can lead to significant reduction of HIV-1 GFP infection in primary human monocyte-derived macrophages (MDM). Furthermore, the mutants are defective in a cell line specific way with certain cell lines being fully permissive to mutant viruses but other cell lines being rather non-permissive to the mutants as compared to wild type virus. We have also assessed whether the mutations impact on the ability of HIV-1 to replicate in human macrophages and whether mutation impacts on the ability of HIV-1 to avoid activating pattern recognition and interferon signalling in these cells. Our data indicate that HIV-1 CA is an important determinant of HIV-1 infectivity impacting on the ability to infect naturally non-dividing targets of HIV-1 infection as well as cell lines in a cell line specific way.

ROLE OF CELLULAR COFACTORS IN PPXY DEPENDENT BUDDING

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Efficient retroviral budding is crucially dependent on the presence of L-(late) domains, short peptide motifs in Gag proteins that recruit components of the ESCRT (endosomal sorting complex required for transport) machinery. Ultimately, these interactions lead to the promotion of viral budding by engaging the membrane scission activity of ESCRT-III. One of these motifs, the PPXY L-domain present in viruses such as MLV (murine leukaemia virus) and HTLV-1 (human T-cell leukaemia virus 1), accesses the ESCRT pathway via interaction with HECT ubiquitin ligases. However, it remains unclear how recruitment of the ESCRT-III complex takes place in this context. In this project, arrestin-like proteins (ARRDC1, 2, 3 and 4 (arrestin domain-containing) were tested for their ability to function as cellular adaptors between HECT Ubiquitin ligases and the ESCRT pathway in PPXY L domain mediated viral budding. We were able to show that arrestin-like proteins interact with both HECT Ubiquitin ligases (WWP1, WWP2 and Itch) and ESCRT components (ALIX and Tsg101). The involvement of this family of proteins in ESCRT mediated processes was supported by our finding, that ARRDC1 colocalises with a catalytically inactive form of the ESCRT component Vps4, a hallmark of proteins interacting with this pathway. Furthermore, we gained insights in the function of arrestin-like proteins by analysis of MLV particle production. These experiments revealed a PPXY specific reduction of infectious particle production in cells transiently expressing ARRDC1, ARRDC2 and ARRDC3. In support of a role in viral egress, confocal analysis showed that ARRDC1 could be recruited to the plasma membrane upon expression of MLV Gag. Together, these results support the hypothesis that arrestin-like proteins could be involved in the recruitment of the ESCRT machinery in PPXY mediated budding.

IS SP1 A SWITCH CONTROLLING HIV-1 PARTICLE ASSEMBLY ?

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Retrovirus assembly is triggered by a nonspecific interaction between Gag and nucleic acids (NA). This is indicated both by encapsidation of cellular mRNAs into psi(-) virions and by the requirement for NA for assembly in a defined system in vitro. The NA requirement is, however, bypassed if the NC domain (the principal NA-binding domain of Gag) is replaced by a leucine-zipper oligomerization domain, raising the possibility that oligomerization of full-length Gag on NA leads to exposure of new interfaces for Gag-Gag interaction and thus to particle assembly. These new interfaces are very likely within the CA domain. The NC and CA domains in HIV-1 Gag are separated by a short linker region called SP1. It has been suggested that the N-terminal part of SP1 is α-helical, but NMR studies show that it has only a slight tendency to form helices in an aqueous solvent. On the other hand, it does form an α-helix in 30% trifluoroethanol (TFE). We have characterized SP1 in several ways. Extensive mutational studies show that proper assembly is exquisitely sensitive to changes in the sequence of the first 6-7 residues of SP1; for example, while the 4th residue (Met) can be replaced with Phe, replacement with Tyr completely destroys the ability of Gag to assemble correctly in 293T cells. Circular-dichroism measurements on free SP1 show that it does indeed undergo a concerted shift to a helical conformation as the TFE concentration is raised. To reconcile all of these observations, we propose the following model: SP1 is a random coil in free Gag, but when Gag oligomerizes, either on NA or via a leucine-zipper motif, the high local protein concentration creates a more nonpolar environment. This environment induces helix formation in the SP1 domain. This change is then “propagated” into the CA domain, leading to exposure of new interfaces and assembly. Various approaches to test this hypothesis are now under way.

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SIVmac VPX PROTECTS HIV-1 FROM THE TYPE I INTERFERON RESPONSE

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Vpx is an accessory protein found in the HIV-2/SIV_{sm}/SIV_{mac} lineage of primate lentiviruses and plays an important role in infection of non-dividing cells such as macrophages and dendritic cells. Pretreatment of monocyte-derived dendritic cells (moDC) with SIV_{mac251}-virus-like particles containing Vpx (Vpx-VLPs) also enhances HIV-1 transduction. Here we extended these observations by examining the effect of Vpx-VLPs on HIV-1 infection in the presence of a type I interferon response. Following establishment of an antiviral state by treatment with type I interferon or pathogen recognition receptor (PRR) agonists, HIV-1 transduction of moDCs was nearly undetectable. Strikingly, despite the establishment of an antiviral state, Vpx-VLPs rescued HIV-1 transduction up to 1000-fold but not HIV-2 or SIV_{mac}. By producing HIV-1 virions in the presence of Vpx and no other SIV factors, Vpx was shown to be sufficient for HIV-1 to overcome the antiviral state. In the absence of interferon, the boost to HIV-1 infection of moDCs by Vpx-VLPs was associated with an increase in nascent HIV-1 cDNA. The larger boost to HIV-1 infection of interferon-treated moDCs by Vpx-VLPs was associated with minimal additional increase in viral cDNA or 2-LTR circles; this suggests that the major effect of Vpx is executed within the target cell nucleus. Vpx-VLPs did not globally block the induction of interferon or of interferon-stimulated genes (ISGs), suggesting that the mechanism involves Vpx-mediated suppression of a particular interferon-induced activity. Vpx mutants that disrupt interaction with VprBP/DCAF1 and knockdowns of this protein in macrophages did not abrogate the observed effect suggesting a Cullin4 E3 ubiquitin ligase independent mechanism. This complex has been reported to be essential for the enhanced HIV-1 transduction mediated by Vpx-VLPs in moDCs and macrophages. In ongoing experiments we are attempting to understand the mechanism by which Vpx rescues HIV-1 from the antiviral state.

STRUCTURE-FUNCTION RELATIONSHIP OF FELINE IMMUNODEFICIENCY VIRUS (FIV) RNA PACKAGING DETERMINANTS

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FIV is a complex retrovirus belonging to the lentiviral genera that causes a disease similar to human acquired immunodeficiency syndrome in domesticated cats. FIV is a potential small animal model for AIDS, and FIV based vectors are also being pursued for human gene therapy. Recently, a series of studies have suggested that similar to the packaging determinants of the primate lentiviruses, human and simian immunodeficiency virus (HIV and SIV), and the packaging determinants of this non-primate lentivirus are complex and multipartite. Specifically earlier studies from our laboratories have mapped the FIV packaging signal to two or more discontinuous regions within the 5' end 511 nucleotides (nt) of the genomic RNA (Mustafa et al., 2005, *J Virol*, 79:13817-21), and structural analyses have determined its secondary structure (Kenyon et al., 2008, RNA, 14:2597-608). Using minimal free-energy structural predictions, biochemical probing, and phylogenetic analyses, we have demonstrated five conserved stem loops (SL1-SL5) and a conserved long-range interaction (LRI) between complementary heptanucleotides in R/U5 (nt 289 5' CCCUGUC 3' nt 295) and Gag (nt 644 3' GGGACAG 5' nt 638). In addition to the LRI, we have identified in stem loop 5 a prominent 10 bp (nt 657 5' AAUGGCCAUU 3' nt 666) palindromic (pal) sequence within the Matrix coding region of Gag that may act as a viral dimerization initiation site. To establish the biological significance of different structural components of the proposed structure and to provide functional evidence for the existence of the LRI, and the role of pal in FIV RNA packaging, we introduced a series of mutations including deletions/substitutions and/or compensatory mutations in the proposed RNA secondary structure. These mutations were tested in a biologically relevant *in vivo* packaging and transduction assay to determine their effects on packaging and replication efficiency of FIV transfer vectors. The detailed results of the deletion and substitution mutational analysis will be presented, which will further validate the RNA secondary structure of the FIV packaging signal proposed by us earlier and establish the biological significance of different structural components in FIV RNA packaging.

THE ABILITY OF SEMEN TO ENHANCE HIV INFECTIVITY CORRELATES WITH ENDOGENOUS LEVELS OF SEVI.

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SEVI (Semen-derived Enhancer of Viral Infection) was identified from a peptide library of pooled human semen as a factor that can greatly enhance HIV infection. SEVI corresponds to an amyloidogenic peptide fragment from prostatic acid phosphatase (PAP), an abundant protein in semen. The amyloid form of this peptide can enhance HIV infection up to 105-fold under conditions of limiting viral inocula. Although we have previously shown that synthetic versions of SEVI can enhance HIV infection, the extent to which endogenous SEVI contributes to the ability of semen to enhance HIV infection was unclear. To address this question, we generated antibodies against SEVI fibrils, and used them to probe relative SEVI levels in individual semen samples obtained from multiple donors. We observed a statistically significant correlation between the viral enhancing activity of semen and anti-SEVI reactivity. Conversely, the enhancing activity of semen was not correlated with levels of prostate specific antigen (PSA) or other proteins present in semen. Furthermore, when semen was depleted of HIV enhancing activity by incubation for prolonged periods at 37oC or by resin-mediated depletion of cationic proteins, we observed a reduction in anti-SEVI reactivity. These findings suggest that endogenous SEVI likely plays an important role in the ability of semen to enhance HIV infection. Microbicides targeting both HIV and SEVI might greatly reduce HIV transmission.

XENOTROPIC MULV-RELATED LEUKEMIA VIRUS (XMRV) IS INHIBITED BY INTERFERON INDEPENDENTLY OF RNASE L OR TETHERIN

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Recent studies suggest that a novel human retrovirus, XMRV, is associated with prostate cancer or chronic fatigue syndrome (3,4). The virus was found in prostate tumors of patients carrying a germline mutation of the HPC1 gene locus, which encodes the antiviral gene RNase L. Previous studies have suggested that XMRV is susceptible to the Interferon (IFN)-induced antiviral state in DU145 prostate cells, but not in LNCaP prostate cells since these cells are deficient in IFN signaling. Reduction in RNase L by RNA interference (RNAi) slightly enhanced XMRV replication in the presence of IFN but had no effect in the absence of IFN (1). Moreover, recent studies indicated that well characterized IFN-induced retroviral restriction factors, such as tetherin and APOBEC3G, are capable of restricting XMRV replication (2).

Despite these observations, it is still unknown what cellular factor is restricting XMRV in the context of an IFN induced antiviral state. To explore possible factors that might restrict XMRV, we reconstituted an IFN antiviral state in an IFN-signaling competent cell line (2fTGH cells - human fibrosarcoma) and tested the ability of XMRV to establish a spreading infection. XMRV replication and spread was completely blocked following IFN exposure, but preceded normally in cell lines deficient in various components of the IFN signaling pathway. Reduction of RNase L steady state protein levels by more than 90% failed to relieve the block imposed by IFN, suggesting that RNase L is not a limiting factor in the context of IFN. The same results were seen upon knockdown of tetherin, an IFN-induced gene product shown to have broad antiviral properties against retroviruses. Further investigations revealed that IFN blocks XMRV replication at early stage in the life cycle and it therefore remains possible that either an unknown factor or multiple known IFN-induced genes are responsible for inhibition of XMRV.

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DEFINING HIV UNCOATING KINETICS, DIFFERENT ASSAYS REVEAL DIFFERENTIAL OUTCOMES OF STABILITY CLASSIFICATION.

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During viral maturation the HIV-1 virion typically forms an electron-dense conical core that houses the viral genome and replication proteins required for the subsequent round of infection. The outer surface of this core is the viral capsid which is compromised solely of the protein CA. Proper formation of this capsid is required for the virus to be infective. Studies of HIV-1 capsids composed of mutated CA proteins have found that mutations increasing or decreasing overall stability of the capsid have strong negative effects on viral infectivity. These findings have led to the theory that the HIV-1 capsid exists in an optimized “metastable” state that is required for full infectivity.

Following fusion with the target cell, the viral capsid begins a process of disassembly that has been termed “uncoating”. Despite significant research over many years, the process of uncoating remains poorly understood. Mutations in CA or host cell restriction factors such as TRIM5α may alter the natural process of uncoating and decrease viral infectivity. The majority of mutations in CA can be classified as mutations that enhance capsid stability (hyperstable) or decrease capsid stability (hypostable). Host factors such as cyclophilin A (CypA) are also considered to play a role in HIV-1 uncoating but the manner in which these proteins function is also not well characterized.

Various assays can be utilized to determine the relative stability of a given HIV-1 capsid. Utilizing a panel of CA mutants we tested several uncoating assays to determine if variances in outcomes would occur between individual panel members depending in the assay chosen. We noted that certain CA mutants had divergent behaviors depending upon the assay utilized to determine the degree of uncoating. Based upon these observations we conclude that the definition of a given CA mutant as hyper- or hypostable is assay-dependent and the choice of assay used to make this determination can alter interpretation of experimental results.

ANALYSIS OF GAMMA RETROVIRUS INTEGRATION BY DEEP SEQUENCING

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The mechanism of gamma retrovirus integration site selection is not only an interesting scientific question, but also a topic of interest to the field of gene therapy. Integration of therapeutic genes into a host genome can and has led to adverse events in multiple therapeutic gene transfer studies. Both fields would benefit from an increase in knowledge of the interaction between the host cell and the gamma retrovirus pre-integration complex. In this study, we extensively analyze the integration site preferences of two gamma retroviruses, murine leukemia virus (MLV) and xenotropic murine leukemia virus-related virus (XMRV) in primary human CD4+ T lymphocytes. Generating these data sets in T lymphocytes will allow us to use bioinformatic methods to compare the integration site data set to previously published ChIP-Seq data with an exact match of cell type. As a result, our bioinformatic methods will be allow us to correlate certain genomic features to increased or decreased integration frequency, possibly leading to the identification of new integration site trends.

EFFECT OF HIV-1 NC PROTEIN ON TAR RNA HAIRPIN OPENING AND CLOSING KINETICS STUDIED BY SINGLE-MOLECULE RNA STRETCHING

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In this work we study the effect of HIV-1 nucleocapsid protein (NC) on the opening and closing kinetics of the TAR RNA hairpin by applying force to the ends of individual RNA molecules using optical tweezers. The TAR hairpin opens up as it is pulled by the ends and closes as it is relaxed, and the forces at which each transition occurs depend on the pulling/relaxation rate. Surprisingly, we find that in the presence of saturating NC, the average TAR opening force increases, and the effect is greater at higher pulling rates. However, extrapolation to zero force shows that the force-free opening rate is ~1000-fold greater in the presence NC. This result suggests that NC decreases the free energy barrier for TAR opening by ~4 kcal/mol. Moreover, we estimate that whereas 12 bp (out of 24 bp total) have to be unzipped by force to completely unfold the hairpin stem in the absence of NC, only about 6 bp must be opened prior to complete unfolding in the presence of NC. These results are consistent with the ability of NC to moderately destabilize DNA bps. While the TAR hairpin remains relatively stable in the presence of NC, its opening rate is significantly increased, illustrating the general nucleic acid chaperone activity of this protein. Future studies aimed at characterization of NC's effect on the TAR closing rate will allow a complete understanding of the effect of HIV-1 NC on the kinetics and equilibrium stability of TAR RNA.

THE ROLE OF KAP1 IN DNA METHYLATION OF ENDOGENOUS RETROVIRUSES

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Intracisternal A-type particles (IAPs) are endogenous retroviral elements that have readily colonized the mouse genome with around two thousand copies. Most of them lie dormant and are lined with silent histone H3K9me3 marks, which we recently identified to be dependent on KAP1, the loss of which leads to the uncontrolled expression of IAP genes in embryonic stem (ES) cells and in early embryos.

Interestingly, IAP elements are also heavily methylated in their DNA and remain so even throughout reprogramming in early embryos when other classes of retroelements are sensitive to genome-wide demethylation. It is thought that sequence-specific signatures maintain continuous methylation of IAPs to keep them silent, although the precise sequences and factors controlling this process are unknown. We propose KAP1 to be implicated since we have observed it to induce *de novo* CpG methylation of a reporter in early embryos.

Our goal is to establish if KAP1 controls DNA methylation of IAP proviruses. We are investigating this question by using bisulphite pyrosequencing to measure the IAP methylation load globally as well as at specific loci. We are comparing DNA taken from embryos that are wild type or knockout for KAP1 to determine if KAP1 is able to protect IAP genomes from DNA demethylation. We are also testing the ability of introduced IAP *cis*-acting sequences to induce *de novo* methylation. These studies will characterise the mechanism of KAP1 silencing of endogenous retroelements during early embryogenesis.

HIV-1 ASSEMBLY ALTERS DYNAMICS AND PARTITIONING OF MEMBRANE COMPONENTS

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Partitioning of membrane proteins into various types of microdomains is crucial for many cellular functions. Tetraspanin-enriched microdomains (TEMs) are a unique type of protein-based microdomain, distinct from membrane rafts, and important for several cellular processes such as fusion, migration, and signaling. TEMs also serve as sites of HIV-1 assembly/egress and tetraspanins regulate HIV-1-induced membrane fusion. It is also well established that HIV-1 particles are enriched in raft lipids. Using different quantitative microscopy approaches, we investigated the dynamic relationship between TEMs, membrane rafts, and HIV-1 exit sites, focusing mainly on CD9. Our results demonstrate that this tetraspanin accumulates at HIV-1 assembly sites as expression of the major viral structural component, Gag, increases. Further, CD9 exhibited confined behavior and reduced lateral mobility at these sites, suggesting that Gag assembly locally traps tetraspanins. In contrast, the raft lipid GM1, while also recruited to budding/assembly sites, exhibited mostly normal mobility in these membrane areas. These results suggest that by actively regulating the local membrane environment at budding sites, HIV-1 creates a membrane milieu favorable for viral assembly and/or transmission to target cells.

DOMAIN SPECIFIC CONTRIBUTIONS TO THE BIOLOGICAL PROPERTIES OF CHIMERIC SUBTYPE B AND C VPU PROTEINS

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Previously, we showed that a subtype C Vpu protein from a clinical isolate of HIV-1 displayed biological properties distinct from those exhibited by a laboratory-adapted subtype B Vpu (HXB2). Inoculation of pig-tailed macaques with a simian-human immunodeficiency virus (SHIV) expressing a subtype C Vpu protein ($SHIV_{SCVpu}$) resulted in a more gradual loss of CD4+ T-cells compared to those inoculated with a SHIV expressing a subtype B Vpu ($SHIV_{KU\text{-}1bMC33}$) (M.S. Hill et al., Virology 371(1):86-97, 2008). In this study, we sought to determine the contributions of the N-terminus/transmembrane domain and the cytoplasmic domain of each of these subtypes to the differences observed. We constructed chimeric Vpu expressing either the subtype B or subtype C N-terminal region/transmembrane domain and the opposing cytoplasmic domain. Both chimeric vpu genes were fused in frame with the gene for enhanced green fluorescence protein (EGFP). Our results indicate that the cytoplasmic domain is responsible for localization of the protein and the difference in molecular weight of the fusion proteins. Both proteins down-regulated CD4 from the surface similar to the SCVpu. We constructed SHIV expressing either of the chimeric Vpu ($SHIV_{VpuBC}$ and $SHIV_{VpuCB}$). $SHIV_{VpuBC}$ replicated with increased kinetics compared to both parental viruses ($SHIV_{KU\text{-}1bMC33}$ and $SHIV_{SCVpu}$) as well as to the $SHIV_{VpuCB}$. $SHIV_{VpuCB}$ replicated with reduced kinetics compared to the $SHIV_{VpuBC}$ and the parental $SHIV_{KU1b\text{-}MC33}$, however at a rate similar if not slightly increased from the parental $SHIV_{SCVpu}$. Inoculation of three pig-tailed macaques with $SHIV_{VpuBC}$ resulted in a rapid loss of circulating $CD4^+$ T-cells and high viral loads. Studies involving inoculation of pig-tailed macaques with $SHIV_{VpuCB}$ are ongoing. These results demonstrate the potential impact of the individual domains of the Vpu protein on biological properties, protein function and pathogenesis.

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STRUCTURAL, BIOCHEMICAL AND BIOPHYSICAL CHARACTERIZATION OF THE INTERACTIONS BETWEEN HIV-1 MATRIX AND CALMODULIN

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HIV-1 encodes a polypeptide called Gag that is capable of forming virus-like particles (VLPs) in vitro in the absence of other cellular or viral constituents. During the late phase of HIV-1 infection, Gag polyproteins are transported to the plasma membrane (PM) for assembly. Gag trafficking and assembly on the PM of the infected host cells are essential to the production of new HIV virions. Great progress has been made in defining both the viral and cellular determinants of HIV-1 assembly and release. However, the trafficking pathway used by Gag to reach its assembly sites in the infected cell is poorly understood. There is mounting evidence that HIV-1 Gag interacts with several cellular proteins during the virus replication cycle and that these interactions are mediated by the MA domain. These include calmodulin (CaM), the suppressor of cytokine signaling 1 (SOCS1), the human adaptor protein-3 complex (AP-3), and tail-interacting protein (TIP47). CaM is a calcium-binding protein expressed in all eukaryotic cells. It can bind to and regulate a number of different protein targets, thereby affecting many different cellular functions. It can have different subcellular locations, including the cytoplasm, within organelles, or associated with the plasma membrane. Previous studies have revealed that CaM co-localizes with Gag in a diffuse pattern in the cytoplasm of infected H-9 cells, Gag-CaM interactions are dependent on calcium, and that these interactions are mediated by the MA domain. Coupled with recent evidence that intracellular calcium stimulates production of virus-like particles, we hypothesize that CaM-MA interactions are probably important for Gag trafficking and assembly. We have employed a set of structural, biochemical and biophysical methods to precisely elucidate the structural requirements of Gag-CaM interactions. Herein, we demonstrate that MA binds directly to CaM in a calcium-dependent manner with a 1:1 stoichiometry and dissociation constant (Kd) of 1.9 μM ± 0.1 μM. NMR, ITC and mutagenesis studies confirmed that MA binds preferentially to the C-terminal hydrophobic pocket of CaM in an entropically driven mode. Furthermore, our data show that the N-terminal myristyl group of MA is not required for CaM binding. We hope that our studies will not only elucidate the structural requirements for Gag-CaM interactions but may establish a potential link between CaM cell signaling pathway and HIV replication and infectivity. Elucidation of the molecular interactions between the host cell and HIV are important for understanding the resulting viral replication and the subsequent cytopathogenesis in the infected cell, which will aid in the development of more efficient antiviral drugs.

INVOLVEMENT OF SUPPRESSOR OF CYTOKINE SIGNALING 1 (SOCS1) PROTEIN IN THE RHESUS MACAQUE TRIM5ALPHA MEDIATED LATE RESTRICTION

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Old world monkey TRIM5α was originally identified as a host factor which restricts HIV-1 infection through recognizing the viral core by its B30.2(PRYSPRY) domain. We have reported that rhesus macaque TRIM5α (TRIM5αrh) also restricts HIV-1 production. The amount of HIV-1 Gag in producer cells was reduced by TRIM5αrh over-expression. TRIM5αrh's encapsidation into virus like particles made by HIV-1 Gag poly-proteins suggested that the target of the late restriction is the Gag protein, not the core structure. Responsible residues for the late restriction locate to the N-terminal region of coiled-coil domain, not in B30.2(PRYSPRY). TRIM5αrh restricts HIV-1 production in a cell line dependent manner, and the study using fused cells among supportive and non-supportive cell lines suggested that TRIM5αrh needs an additional host factor(s) to block HIV-1 production. Here we found that SOCS1 bridges the interaction between TRIM5αrh and HIV-1 Gag protein. SOCS1 is known to enhance HIV-1 production by rescuing HIV-1 Gag protein from lysosomal degradation (Ryo A *et al*. PNAS 2008). When SOCS1 was over-expressed in producer cells, HIV-1 production was rescued from the TRIM5αrh-mediated late restriction. In the presence of SOCS1, Gag poly-protein, but not matured/monomeric CA, co-immunoprecipitated with TRIM5αrh by an α-HA pull-down. Human TRIM5α (TRIM5αhu) did not show the interaction with SOCS1 protein in producer cells, and HIV-1 Gag protein was not obtained by an α-HA (TRIM5α) pull down, even when SOCS1 was over-expressed. Over-expression of SOCS1 reduced the amount of TRIM5αrh, but not TRIM5αhu, in transfected cells. Thus we hypothesized that TRIM5αrh restricts HIV-1 production through an interaction with SOCS1. Although this TRIM5αrh mediated late restriction still remains controversial and further study and discussion about the antiviral activity are required, our findings give new insight into understanding the role of TRIM5α in innate-immunity.

ANALYSES OF THE EFFECT OF NEF-DELETIONS ON SIVCPZGAB2 PROVIRUS

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The viral protein Nef is a pathogenic factor present in Simian and Human Immunodeficiency Viruses (SIV and HIV). The absence of Nef reduces viral infectivity about 4-40 fold and disease progression both in humans and macaques. The mechanisms underlying the gain of infectivity conferred by Nef is still not fully understood. We analyzed the effect of complete and partial deletions of the nef gene for the replicative cycle of the SIVcpzGAB2 infectious clone. Disruption of the Nef ORF was achieved by mutagenesis of the start codon of nef which resulted in the complete absence Nef expression (SIVcpzΔNef), or by insertion of two nucleotides at position 218 which resulted in the expression of a truncated N-terminus of Nef (76aa peptide) (SIVcpz t-Nef). The SIVcpzΔNef was 2-4 fold less infectious than the wild type SIVcpz in TZM-bl cells and, after providing Nef in trans, the infectivity increased to the levels of the wild type virus. Infectivity of the SIVcpz t-Nef was completely abrogated in TZM-bl cells and was not rescued by providing Nef in trans. Analyses of Gag processing showed an accumulation of the p55Gag and the intermediate precursors and a reduction of p24CA levels both in cell lysates and viral particles. Furthermore, the truncated Nef peptide interfered with the infection of SIVcpzΔNef and HIV-1ΔNef. “Chase” assays of 293T transfected cells treated with 10mM of cycloheximide showed that in the absence of Nef the p55Gag had a faster kinetic of processing. These data confirm that Nef is an infectious factor for SIVcpz and suggest that Nef has a correlation with the viral protease during virus particle formation.

EVIDENCE FOR HIV-1 G-to-A HYPERMUTATION IN VIVO BY APOBEC3 PROTEINS

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It has been shown that the hypermutated HIV-1 sequences from infected patients exhibit strong preferences for G-to-A. In 2003, human APOBEC3G was identified as an HIV-1 restriction factor, which edits nascent HIV-1 DNA by inducing G-to-A hypermutations and debilitates the infectivity of *vif*-deficient HIV-1. On the other hand, HIV-1 Vif protein has the robust potential to degrade APOBEC3G protein. Although following investigations have revealed that lines of APOBEC3 family proteins have the capacity to mutate HIV-1 DNA, it remains unclear whether these endogenous APOBEC3s contribute to mutations of *vif*-proficient HIV-1 provirus *in vivo*.

In this study, we use a human hematopoietic stem cell-transplanted humanized mouse (NOG-hCD34 mouse) model and show that Vif is prerequisite for HIV-1 expansion. Notably, we demonstrate the predominant accumulation of G-to-A mutations in HIV-1 provirus displaying characteristics of APOBEC3-mediated mutagenesis in the presence of Vif. It was of interest that the frequency of APOBEC3-associated HIV-1 G-to-A mutations leading to termination codons was significantly observed. Although it is now considered that endogenous APOBEC3s contribute to HIV-1 G-to-A hypermutation in patients, there are no investigations focusing on the influence of APOBEC3s on HIV-1 *in vivo*. Since the original sequence of HIV-1 in an infected individual is inaccessible, the previous observations of HIV-1 mutation in patients were mostly based on comparative analyses to prototypic HIV-1. Therefore, this is the compelling evidence indicating that endogenous APOBEC3s are associated with G-to-A hypermutation of HIV-1 provirus *in vivo*, which can result in the abrogation of HIV-1 replication.

DEVELOPMENT AND EVALUATION OF HEPATITIS DELTA VIRUS RZS TARGETING HIV RNA

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The use of gene transfer as a means to deliver anti-HIV molecules to target cells provides a promising approach to control infection. The use of catalytic RNAs or ribozymes (Rzs) engineered to target HIV RNA is one of several approaches that can be used to generate HIV resistant cells with the potential to inhibit HIV replication. The hepatitis delta virus Rz (HDV-Rz) was the first self-cleaving Rz motif identified which naturally functions in human cells. In order to improve upon the limited target specificity of previous trans acting HDV-Rzs, Dr. Perreault's Laboratory developed The Specific On/oFf Adaptor (SOFA) HDV-Rz motif (1).

Our aim is to evaluate whether SOFA HDV-Rzs could be used to target HIV RNA and inhibit the production of new HIV virus in cells. Towards this aim we have generated SOFA HDV-Rzs targeting HIV RNA and have identified several which can both cleave HIV RNA in vitro and inhibit HIV expression in cell assays. Our results suggest that SOFA-HDV Rzs targeting HIV RNA could be a useful addition to the arsenal of anti-HIV gene therapy agents.

Reference:
1: Bergeron and Perreault. 2005. Target-dependent on/off switch increases ribozyme fidelity. Nucleic Acids Res. 33(4):1240-1248.

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CHARACTERIZATION OF THE RETROVIRAL SILENCING MACHINERY IN EMBRYONIC STEM CELLS

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The pluripotency of Embryonic stem cells (ESC) mandates stringent mechanisms for maintaining genomic integrity, among them the ability to silence potentially genotoxic attacks by endogenous and exogenous retroelements. Although infection of ES and Embryonic Carcinoma (EC) cells by MMLV results in the successful integration of the proviral DNA into the genome, transcription from the viral promoter in the long terminal repeat (LTR) is potently silenced. This restriction is partly due to a large nuclear complex that binds to a conserved DNA element called the primer binding site (PBS). It was recently shown that the PBS of MMLV- PBSpro -binds a DNA binding Zinc-Finger protein called ZNF809, which further recruits TRIM28 (Kap1), a known transcriptional silencer. TRIM28 recruits several factors involved in transcriptional silencing and heterochromatin formation.

The remarkable ability of ESC to potently suppress retroviruses constitutes a unique opportunity to decipher the molecular mechanisms underlying gene silencing used by these cells. Thus, in order to characterize the silencing machinery over time and at various developmental stages, we are using a reporter system that enables us to measure viral restriction in a live population of cells. In this system, MMLV LTR-PBS sequence is fused to GFP and is then used to infect cells. Repression mediated by the PBS can be relieved by replacing the PBSpro with a sequence complementary to glutamine (Gln) tRNA, and this PBSgln is used as our control virus. Using these viruses we show a high PBS-mediated restriction rate in EC and ES cells. However, when we knock down ZFP809 we see no PBS-mediated repression at all. This suggests that the PBS restriction mechanism plays a major role in the ESC specific silencing of viral expression. Strikingly, the silencing machinery is active immediately after viral DNA integration and is retained at the same efficiency for weeks. Moreover, even the small sub-population of cells that escape silencing in a defined window of time are again silenced after one day. Interestingly, in cells infected with PBSgln or in ZFP809 KD cells the restriction efficiency of ES and EC cells is much higher than that seen in somatic cells. This implies the existence of an alternative silencing mechanism. Finally, we are mapping the correlation between differentiation and viral repression by looking at cells in different stages of development.

These studies provide us with a better understanding of the specific features of the stem cells that allow for their genetic stability and resistance to viral replication.

INVESTIGATING THE FUNCTIONAL ROLE OF THE NUCLEOLUS IN HIV-1 VIRAL RNA EXPORT

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Over the past decade, it has been established that the nucleolus is not just a site for ribosomal biogenesis; it is a multifunctional compartment involved in other processes such as cell cycle regulation, cell stress response, the processing and maturation of non-ribosomal RNAs, and viral replication. Distinct steps in viral RNA (vRNA) replication have been shown to occur in the nucleolus for both the Borna disease virus and the hepatitis delta virus. Picornaviruses and hepatitis C virus interact with the nucleolar protein nucleolin to stimulate translation. The herpesvirus saimiri nucleocytoplasmic shuttling protein ORF57 localizes to the nucleolus, recruits transcription/export proteins to the nucleolus, and is required to traffic through the nucleolus for vRNA transport.

The HIV-1 regulatory protein Rev has also been demonstrated to localize to the nucleolus. Rev mediates the nucleocytoplasmic transport of intron-containing vRNA by binding to a stem-loop structure, the RRE, and recruiting CRM1-dependent export machinery. It has been reported that Rev can induce the relocalization of CRM1, Nup98 and Nup214 to the nucleolus, suggesting formation of Rev:factor export complexes within the nucleolus. Furthermore, Rev can multimerize in the nucleolus and can actively bind and export nucleolar localized RRE-containing target RNAs. Collectively, these finding suggest that the nucleolus may have a functional role in the nuclear export and/or subsequent translation of Rev-directed RNAs. Nonetheless, whether this nucleolar localization plays a role in HIV-1 vRNA export or expression remains controversial. Initial steps towards elucidating this role, we have analyzed the intracellular distribution of vRNA from proviral clones containing select mutations in Rev or the RRE using coupled fluorescence *in situ* hybridization followed by indirect immunofluorescence. Using a fluorescent resonance energy transfer assay, we have also analyzed the functional interactions of nucleolar proteins, previously identified as Rev cofactors, during Rev-mediated export of RRE-containing vRNA from the nucleus to the cytoplasm.

UNIQUE DETERMINANTS OF RHESUS APOBEC3F THAT ALLOW FOR INCREASED VIRION INCORPORATION AND DECREASED VIRAL RESTRICTION.

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Human APOBEC3G (hA3G) and APOBEC3F (hA3F) exhibit potent anti-HIV-1 activity by restricting retroviral infection through the deamination of cytidine residues during minus strand DNA synthesis. Our studies indicate that rhesus APOBEC3G (rhA3G) is not incorporated into wild-type pathogenic simian-human immunodeficiency virus ($SHIV_{KU-2MC4}$) while rhesus APOBEC3F (rhA3F) is readily incorporated. This result suggests that SIV Vif may not directly interact with rhA3F. In order to examine the determinants involved, we constructed vectors expressing human and rhesus A3F proteins in which the C-terminal domains were exchanged. Introduction of the rhesus C-terminal domain into hA3F resulted in decreased virion incorporation and viral restriction. We next sought to identify the specific residues responsible for this inhibition of function as well as determine if rhesus and/or pigtailed macaques expressed naturally occurring variants of APOBEC3F efficient in neutralizing Vif-deficient viruses. The rhA3F genes from five rhesus and pigtailed macaques were cloned and sequenced. Viral properties of each clone were assessed using infectivity, incorporation, and hypermutation assays. We found that the C-terminus of rhesus APOBEC3F contains unique amino acid residues critical for APOBEC3F virion incorporation and decreased viral restriction. The results from this study further indicate that SIV Vif is a less potent inhibitor of rhA3F.

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THE NEVIRAPINE RESISTANCE MECHANISM OF N348I HIV REVERSE TRANSCRIPTASE IS BASED ON INCREASED DISSOCIATION OF THE INHIBITOR

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We previously identified clinical isolates with phenotypic resistance to the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) in the absence of known NNRTI mutations. We showed that this resistance is caused by N348I, a mutation in the connection domain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) which confers dual-class resistance to NNRTIs (NVP) and NRTIs (AZT). N348I has been shown to be a common mutation with a prevalence of 10% among patients treated with antiretroviral therapy and <2% among untreated HIV patients.

To understand the effect of N348I on the biochemical functions of HIV RT we carried out detailed characterization of enzymes containing the N348I mutation. Using subunit-specific mutagenesis we constructed enzymes with the N348I mutation in the p66 (p66N348I/p51WT), the p51 (p66WT/p51N348I), or in both subunits (p66N348I/p51N348I, or N348I) of RT. RNase H activity assays with these enzymes indicate that the previously reported changes in RNase H activity are the result of the mutation in p51. However, changes in both subunits affect susceptibility to nevirapine. N348I RT is more processive than the WT enzyme and this processivity is affected by mutation in either subunit. Pre-steady state kinetics analysis demonstrated that N348I moderately enhances nucleic acid binding. However, transient kinetics experiments revealed that N348I has impaired efficiency of incorporation as compared to WT due to reduced nucleotide incorporation rate (k_{pol}). The efficiency ratio (k_{pol}/ $K_{d,dNTP}$) for WT was ~4 fold higher than that for N348I RT.

The kinetics of NVP binding were determined through the measurement of $K_{d,NVP}$ and observation of $k_{app,dNTP}$ in the presence of NVP. N348I displayed an approximately 3-fold increased $K_{d,NVP}$ (decreased binding) relative to WT. An increase in the dissociation constant $k_{off,NVP}$ for N348I was responsible for the difference in $K_{d,NVP}$ values, while the association rates ($k_{on,NVP}$) of the WT and N348I mutant were similar.

These findings provide the basis for understanding the role of connection subdomain mutations in NNRTI resistance. They also expand the previously accepted structural support role of p51 to include modulation of diverse biological functions including binding affinity for substrates, catalytic turnover, polymerase and RNase H activities, and drug resistance.

THE ROLE OF ALIX-NEDD4-1 INTERACTION IN HIV-1 RELEASE

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In addition to the PTAP-type L domain, HIV-1 Gag contains a second L domain motif, LYPXnL, that promotes virus release from both 293T and T cells. LYPXnL interacts with the cellular protein Alix to gain access to the host ESCRT-III proteins and facilitate virus separation from the cell. We recently described a new interaction between the ESCRT-associated protein Alix and the Nedd4-like ubiquitin ligase Nedd4-1, and showed that this interaction is important for HIV-1 release mediated via the Alix-binding LYPXnL L domain motif. To further characterize this interaction and its role in HIV-1 release, we examined the nature of the ubiquitin-modification of Alix. We found that Alix is mostly monoubioquitinated in the cell. Moreover, mono-ubiquitinated Alix is incorporated in HIV-1 particles whose release was triggered with the ectopic expression of Alix, suggesting that Alix is ubiquin-modified in late stages of HIV-1 release from the plasma membrane. Additionally a short motif in the N-terminal portion of Nedd4-1, contained within a region known to mediate the peripheral binding of Nedd4-1 to membranes, was also found to be critical for Nedd4-1's facilitation of HIV-1 release. Mapping of regions in Alix that interact and are ubiquitinated by Nedd4-1 has been conducted, and the significance of such modification in Alix's facilitation of HIV-1 release will be presented and discussed.

UP-REGULATION OF ANTI-APOPTOSIS AND DOWN-REGULATION OF DNA DAMAGE RESPONSE MARKERS IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY VIRUSES CONTAINING K65R AND MDR MUTATIONS IN COMPARISON TO WILD TYPE VIRUS

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Clinical trials and *in vitro* cell culture studies have elucidated the beneficial role that replication disadvantaged viruses may play in reducing HIV viral loads. Ex-vivo studies have demonstrated that wild type HIV-1 induces direct apoptosis of infected cells; however, the contribution of less fit viruses in T-cell apoptosis is not clear. A fundamental issue is whether less fit viruses can confer variable virulence and effect $CD4^+$ T-cell depletion differently than wild type viruses. Since $CD4^+$ T-cell apoptosis is a dynamic process and involves a series of cytokines/chemokines and –pro and –anti apoptosis inducing proteins, we hypothesized that mutated viruses will up-regulate the expression of anti-apoptosis marker proteins and down regulate the expression of DNA damage response proteins in comparison to wild type viruses.

We performed a RT-PCR-based apoptosis-array analysis on total cellular RNA isolated from PBM cells infected with wild type (NL4-3) and mutated viruses containing RT mutations K65R, and clinical RT containing multiple mutations (D67N, S68G, K70R, K103N, T215Y, K219E). Apoptosis-array based RT PCR (SA Biosciences, MD) allowed us to compare the expression of 90 apoptosis-related genes. Analysis of data showed that several anti-apoptosis genes were in fact expressed 2 to 10 –fold higher in mutated viruses in comparison to wild type virus. We found anti-apoptosis genes BAG1, BIRC6, BRAF, MCL1, TNF and CD27 were up-regulated 2 to 4 –fold in the PBM cells infected with viruses containing K65R and MDR mutations in comparison to the cells infected with WT-NL4-3 viruses. We also found that genes related to p53 and DNA damage response, AKT1, APAF1, BAX, BCL2L1, CASP7, TP53 were 2 to 6 –fold down regulated in cellular RNA derived from PBM cells infected with K65R viruses in comparison to WT viruses.

Based on these observations, it is possible that replication disadvantaged viruses could alter apoptosis and reduce $CD4^+$ T-cell depletion associated with HIV disease.

THE HIV-1 DIMERIZATION INITIATION SITE (DIS) IS INVOLVED IN THE REGULATION OF VIRAL RNA SPLICING AND PACKAGING

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During the packaging process, HIV-1 genomic RNA (gRNA) is preferentially selected amongst a multitude of cellular and viral spliced mRNAs. As for all retroviruses, RNA is incorporated into viral particles as a dimer of two homologous molecules. Packaging of HIV-1 gRNA involves specific interactions between a trans-acting factor, the Gag precursor, and cis elements present in the leader region of the gRNA (packaging signals). This region located at the 5'-end folds into four stem-loop structures, SL1 to SL4, involved in several important steps of the HIV-1 life cycle (RNA dimerization and packaging, splicing, initiation of translation).
The location of the HIV-1 Dimerization Initiation Site (DIS or SL1), immediately upstream of the SD site, results in its presence in all spliced viral mRNAs. The DIS in the context of HIV-1 spliced mRNAs is fully functional in vitro but does not promote their packaging, while it is a major element for gRNA encapsidation. Moreover, the fact that RNA dimerization takes place during in vitro transcription suggests it could affect the fate of HIV-1 RNAs by modulating splicing, transport or/and localization.
In order to determine if RNA dimerization and splicing could be related, we either mutated the DIS or/and moved it away from the SD. We then analyzed the yield of viral RNAs in transfected 293T cells and in viral particles by quantitative RT/PCR and evaluated the importance of the DIS and SD proximity on splicing and RNA packaging. We found that all mutations affecting either the DIS or the distance between the DIS and the SD site decreased gRNA packaging efficiency, while multi-spliced viral RNAs were slightly increased. Interestingly, we observed a 2-3 fold increase in splicing efficiency when the DIS was disrupted or displaced away from the SD. These results suggest that the DIS and/or RNA dimerization could possibly regulate viral RNA splicing and gRNA selection.

EXPRESSION OF NEF FROM UNINTEGRATED HIV-1 CDNA DOWNREGULATES CELL SURFACE MHC-I, CXCR4 AND CCR5

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Transcription of HIV-1 cDNA prior to, or in the absence of, integration leads to synthesis of all classes of viral RNA transcripts. Yet only a limited range of viral proteins, including Nef, are translated in this context. Nef expression from unintegrated HIV-1 cDNA has previously been shown to reduce cell surface CD4 levels in T-cells. We wished to determine whether Nef expressed from unintegrated cDNA is also able to downregulate MHC-I as well as the chemokine coreceptors CXCR4 and CCR5.

Infected cells bearing unintegrated HIV-1 cDNA were assayed in the GFP reporter cell line Rev-CEM for cell surface levels of HLA-A2, HLA-ABC, CXCR4, CCR5 and CD4. Viral integration was blocked either through use of an inactive integrase, or through the use of the integrase inhibitor raltegravir. Reverse transcription, integration and the proliferation of unintegrated DNA were confirmed by PCR.

In cells bearing only unintegrated HIV-1 cDNA we found that cell surface levels of HLA-A2, HLA-ABC, CXCR4 and CCR5 were significantly reduced, and we also confirmed the cell surface down-regulation of CD4. Similar patterns of results were obtained with both integrase-deficient virus or with wild type virus where integration was blocked by raltegravir.

Our results demonstrate that Nef can be expressed from unintegrated cDNA at functionally relevant levels, and suggest that the downregulation of MHC-I and entry receptors by Nef in this manner could aid immune evasion, restrict superinfection and prevent signal transduction involving HIV-1 infected cells.

P BODY-ASSOCIATED PROTEIN MOV10 IS INTERFERON-INDUCIBLE AND INHIBITS HIV-1 REPLICATION AT MULTIPLE STAGES

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Recent studies have shown that APOBEC3G (A3G), a potent inhibitor of HIV-1 replication, is localized to cytoplasmic mRNA-processing (P) bodies. However, the functional relevance of A3G colocalization with P body marker proteins has not been established. To explore the relationship between HIV-1, A3G, and P bodies, we analyzed the effects of overexpression of P body marker proteins Mov10, DCP1a, and DCP2, and DDX6 on HIV-1 replication. Our results show that overexpression of Mov10, a putative RNA helicase belonging to the DExD superfamily, leads to potent inhibition of HIV-1 replication at multiple stages. In contrast, overexpression of the decapping enzymes DCP1a and DCP2, or the RNA helicase enzyme DDX6 did not inhibit HIV-1 replication. Mov10 overexpression in the virus producer cells resulted in reductions in the steady-state levels of HIV-1 Gag protein and virus production. Mov10 was efficiently incorporated into virions and reduced virus infectivity, in part by inhibiting reverse transcription. In addition, A3G and Mov10 overexpression modestly reduced proteolytic processing of HIV-1 Gag. Quantitation of the amounts of cellular gag and gag released from cells in the presence of Mov10 overexpression indicated that there was no apparent defect in virion release. The inhibitory effects of A3G and Mov10 were additive, implying a lack of functional interaction between the two proteins. siRNA-mediated knockdown of endogenous Mov10 by 80% did not significantly affect viral replication, suggesting that endogenous Mov10 was not required for viral infectivity. Intriguingly, Mov10 expression in $CD4^+$ T cells was suppressed upon phytohemeagglutinin activation and strongly induced in the activated cells by as much as 41-fold upon treatment with interferon-γ. There was no induction of Mov10 in macrophages with interferon treatment. The levels of Mov10 expressed in transfected 293T cells were comparable to those present in interferon treated $CD4^+$ T cells. Overall, these results show that Mov10 is an interferon-inducible protein that can potently inhibit HIV-1 replication at multiple stages.

SUSCEPTIBILITY OF XMRV TO ANTIRETROVIRAL INHIBITORS

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Xenotropic murine leukemia virus-related virus (XMRV) is a gammaretrovirus that was originally discovered in a microarray analysis of prostate tumor tissues (Urisman et. al., PLoS Pathog. 2006) and has recently been detected in a cohort of patients with chronic fatigue syndrome. Previous studies suggest that XMRV is susceptible to AZT in culture but is resistant to certain protease inhibitors, integrase inhibitors and the nucleoside analogs 3TC, D4T and PMPA (tenofovir). In the present study, we used a single-cycle indicator cell assay to directly compare the sensitivities of HIV-1 and XMRV to a panel of antiretroviral drugs. Our analysis included two independently isolated strains of XMRV: $XMRV_{VP62}$, which was produced from a full-length infectious molecular clone, and $XMRV_{22Rv1}$, an isolate obtained from a prostate carcinoma cell line. With regard to nucleoside RT inhibitors (NRTIs), $XMRV_{VP62}$ and $XMRV_{22Rv1}$ were susceptible to AZT, PMEA (adefovir) and PMPA in culture, but were 13–29-fold resistant to ddI, D4T and ABC (abacavir) and >100-fold resistant to 3TC and FTC relative to HIV-1_{NL4-3}. $XMRV_{VP62}$ and $XMRV_{22Rv1}$ were additionally >20-fold and >200-fold resistant to the nonnucleoside RT inhibitors nevirapine and efavirenz, respectively. We also measured the sensitivity of $XMRV_{VP62}$ to each of nine protease inhibitors (PIs) that are active against HIV-1 in vitro and in vivo. Concentrations of PIs that were 10–40-fold greater than the EC_{50} for HIV-1_{NL4-3} failed to inhibit $XMRV_{VP62}$ infection in our assay system, indicating high-level resistance to this particular drug class. However, the integrase inhibitors raltegravir and elvitegravir demonstrated potent activity against both $XMRV_{VP62}$ and $XMRV_{22Rv1}$, with EC_{50}s in the sub-micromolar range. Taken together, our results demonstrate that XMRV exhibits a distinct pattern of NRTI sensitivity that correlates with the structure of the pseudosugar moiety, and that XMRV is susceptible to a broader range of antiretroviral drugs than was previously appreciated. Our findings carry important implications for the potential use of antiretroviral drugs for treating XMRV infection.

IDENTIFICATION OF SPECIFIC DETERMINANTS IN APOBEC3F THAT INTERACT WITH HIV-1 VIF

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Human APOBEC3F (A3F) and APOBEC3G (A3G) cytidine deaminases are potent host restriction factors, which suppress retroviral replication by hypermutating the viral genome, inhibiting reverse transcription, and inhibiting viral DNA integration. To overcome A3F- and A3G-mediated inhibition, HIV-1 encodes Vif, which binds and targets these proteins for proteasomal degradation. Previously, we reported that the A3F-Vif interactions that lead to A3F degradation are distinct from the A3G-Vif interactions and are located near the C-terminal end of A3F between amino acids 283-300*.

To further elucidate the A3F-Vif interactions, we performed extensive mutational analysis of A3F and analyzed the mutants for their effects on antiviral activity, sensitivity to Vif-induced degradation, and co-immunoprecipitation assays. Our results show that A3F amino acids ^{289}EFLARH^{294} are critical for binding to Vif and for sensitivity to Vif-mediated degradation. Mutation of one or more amino acids in this region can decrease A3F protein degradation and significantly increase A3F's ability to inhibit viral infectivity in the presence of Vif. Furthermore, coimmunoprecipitation assays showed that Vif binding to mutants with changes in these amino acids can be significantly decreased. We also identified other A3 proteins which have the EFLARH sequence, including human A3C, human A3DE, African green monkey A3F, and rhesus macaque A3F, and examined the role of this site in the interactions between these proteins and HIV-1 Vif. Consistent with previous findings, we observed that human A3C, human A3DE, and African green monkey A3F are all susceptible to degradation induced by HIV-1 Vif, while rhesus macaque A3F is not. Here, we demonstrate that mutagenesis in the EFLARH sites of human A3C, human A3DE, and African green monkey A3F decrease their susceptibilities to Vif-induced degradation. Together, these results indicate that the EFLARH region in human A3C, A3DE, and African green monkey A3F interacts with HIV-1 Vif and that this interaction plays a role in Vif-mediated proteasomal degradation. However, other determinants in rhesus macaque A3F are important for interaction with HIV-1 Vif. These studies provide structural insights into the A3F-Vif interactions that could facilitate the development of a novel class of anti-HIV agents.

*Russell, R.A., Smith, J., Barr, R., Bhattacharyya, D., and Pathak, V.K. (2009) J. Virol. 83:1992-2003.

A NOVEL CELL SURFACE RECEPTOR THAT WAS USED BY AN ANCIENT PRIMATE RETROVIRUS

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The resurrection of inactive endogenous retroviruses allows us to learn about interactions between extinct pathogens and their hosts that occurred millions of years ago. One such paleovirus, chimpanzee endogenous retrovirus group 2 (CERV2), is a relative of modern murine leukemia viruses that is found in the genomes of a variety of old world primates, but is absent from the human genome. The non-existence of a human CERV2 orthologue is peculiar given the numerous apparent cross-species transmissions that occurred between ancestors of old world monkeys, gorillas, and chimpanzees. In order to determine if the absence of CERV2 from the human genome can be explained by species-specific receptor usage, we constructed a consensus CERV2 envelope protein derived using sequences found in the chimpanzee genome. CERV2 enveloped MLV particles were capable of infecting cell lines from a wide range of species, including humans. The permissivity of human cells for CERV2 argues against the notion that human ancestors were protected from CERV2 by the lack of a functional receptor.
Using a Hela cDNA library expressed in CERV2 resistant hamster cells, we identified copper transport protein 1 (CTR1) as a novel retrovirus receptor that was presumably used by CERV2 during its exogenous replication more than one million years ago. Expression of human CTR1 was sufficient to confer CERV2 permissively to otherwise resistant hamster cell lines, which was accompanied by an increase in virion binding. The observed increase in hamster cell infection that came with CTR1 expression was specific to CERV2 enveloped virus, with no gain in permissivity to pseudotypes bearing envelopes from several other gammaretroviruses. Furthermore, siRNA-induced CTR1 knockdown, or CuCl2 treatment specifically decreased CERV2 infection of human cells. We have also identified mutations in highly conserved CTR1 residues that have rendered hamster CTR1 inactive as a CERV2 cell surface receptor, including a deletion in a copper-binding motif that is largely conserved from humans to zebrafish. These receptor-inactivating mutations in hamster CTR1 are accompanied by an increased number of extracellular copper-coordinating residues compared to CTR1 proteins from other species. This apparent compensation may represent an evolutionary barrier that primates would have had to overcome to avoid CTR1 usage by CERV2.

DISRUPTION OF THE MOLONEY MURINE LEUKEMIA VIRUS PREINTEGRATION COMPLEX BY VACCINIA-RELATED KINASES

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Integration, an essential step for retroviral infection, is executed by a high-order nucleoprotein complex termed the preintegration complex (PIC) that is composed of a copy of viral DNA together with a number of viral and cellular proteins. Barrier-to-autointegration factor (BAF) is a cellular component of Moloney murine leukemia virus (MoMLV) and human immunodeficiency virus type 1 (HIV-1) PICs, which facilitates the intermolecular integration and blocks autointegtration of the PIC by its DNA-binding activity. Recent report has shown that BAF is phosphorylated by a family of cellular serine/threonine kinases called vaccinia-related kinases (VRKs) and this phosphorylation disables DNA-binding activity of BAF. This data therefore raises a possibility that the VRK-mediated phosphorylation of BAF could abolish function of the PIC through removal of BAF from viral DNA. In this study, we obtained evidence that murine VRK1 indeed induced abolishment of the intermolecular integration activity of MoMLV PICs *in vitro*. Importantly, this was accompanied by an enhancement of autointegration activity and dissociation of BAF from the PICs. Amongst three VRK family members, VRK1 and VRK2, which catalyzed hyperphosphorylation of BAF, inhibited the PIC activity. We also found that treatment of PICs with certain nucleotides such as ATP resulted in the inhibition of the intermolecular integration activity of PICs and the dissociation of BAF. However, the ATP-induced dysfunction of PICs was not observed in the PICs derived from VRK1-knockdown cells, indicating involvement of endogenous VRK1 in regulation of PIC activity. Our results suggest the presence of an intrinsic inhibitory mechanism for the retroviral PIC integration.

LACTIC ACID IS A NATURAL MICROBICIDE WITH HIV VIRUCIDAL ACTIVITY

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Less than one in three women have vaginal microflora dominated by *Lactobacilli*, and these women are at significantly lower risk of acquiring HIV. When *Lactobacilli* dominate the vaginal flora they acidify the vagina to pH~3.8 by maintaining a ~1.0% racemic mixture of D and L isomers of LA. LA has a pKa of 3.8, and at pH 3.8 comprises uncharged LA and lactate anion. We recently discovered that LA inactivates HSV stereo-specifically, with L-LA more potent than D-LA. We hypothesized that LA inactivates HIV. HIV_{Ba-L} was treated with L-LA and incubated with continuous monitoring of pH and temperature. HIV_{Ba-L} was treated with different concentrations of DL-LA, D-LA and L-LA at 37°C for 30 min. Virucidal activity of 1% L-LA (pH □3.8) was compared to low pH alone (pH 3.8, HCl-adjusted) at different times at 37°C. L-LA was further tested against several HIV isolates and in the presence of 75% human seminal plasma (SP) to mimic a 1:4 dilution of LA during coitus. Sodium lactate was evaluated for virucidal activity. Following incubation, buffered medium was added to dilute out LA and virus titre determined in TZM-bl cells. 0.3% L-LA was ~10^2-fold more potent than D-LA in inactivating HIV_{Ba-L}, and was more potent than DL-LA. Similar results were observed with a clade A CCR5 strain. Treatment of HIV_{Ba-L} with 1% L-LA (pH 3.8) resulted in ~10^4-fold reduction in titre within 1 min and was 10^3-fold more potent than pH 3.8 alone (HCl-adjusted). 0.4% L-LA inactivated (>10^3-fold) HIV-1 clades A, EA, C and three clade B patient isolates. L-LA (at 1.0% final concentration) retained maximum HIV virucidal activity in the presence of 75% SP. 1% sodium lactate (pH 7.0) was not virucidal. LA inactivation of HIV, as for HSV, demonstrates stereo-chemical dependence, suggesting a viral protein target, and is rapid, irreversible and more potent than low pH alone. LA (i.e. protonated, uncharged LA) and not the lactate anion is virucidal. LA's HIV virucidal activity in the presence of SP and the ability of LA to inactivate a broad-spectrum of HIV strains (R5, X4 and dual-tropic) suggests LA is a vaginal defense factor against HIV. Our results also suggest that LA has promise as a microbicide that might block female to male, and potentially also reduce male to female transmission of HIV. Microbicides that employ LA might be valuable since most women do not have *Lactobacillus*-dominated vaginal flora and are at greater risk of acquiring HIV.

A NOVEL HIV-1 VIF FUNCTION IS MEDIATED BY INTERACTION WITH ONCOPROTEIN P53

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Background: HIV-1 Vif plays a crucial role in the viral life cycle by antagonizing a host restriction factor APOBEC3G. Vif has been reported to induce G2 arrest and cell death; however, the mechanisms and biological significance of this function remain unclear. Here we demonstrate that p53 pathway is involved in Vif-mediated G2 arrest and cell death.
Methods: We examined the effect of Vif and it's mutants on cell cycle with PI staining and apoptosis with TUNEL assays using several cell lines. We infected CEM-SS T cells with NL4-3 virus possessing wild-type or mutant Vif and measured the concentration of p24 in the supernatant to examine the effect of Vif-induced cell cycle arrest on HIV-1 replication.
Results: NL4-3 Vif overcame the inhibitory activity of MDM2 on p53-mediated transcription in p21- and bax-promoters. These suggest that Vif can enhance p53 stability and transcriptional activity by blocking MDM2-mediated degradation. Vif could also induce G2 cell cycle arrest and apoptosis via the p53 pathway. However, the Vif derived from HXB2 strain doesn't have any these functions. We compared the sequence between NL4-3 and HXB2 Vif and constructed several mutants and tested their effects on cell cycle. We found that several amino acids changes in NL4-3 Vif lost its function for inducing G2 arrest, whereas the opposite changes in HXB2 Vif render it to induce G2 arrest. Finally, we examined the viral replication in CEM-SS T cells infected with NL4-3 wild-type, deltaVif, or mutant viruses deficient for the function to induce cell cycle arrest.
Conclusions: These data imply the functional interaction between Vif and the p53 axis.

HUMAN RETROVIRUS CELL-TO-CELL TRANSMISSION AT VIROLOGICAL SYNAPSES THROUGH “VIRAL BIOFILMS”

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For many retroviruses, direct cell-to-cell transmission appears to be much more efficient than spread via free viral particles. The case of Human T cell leukemia virus type 1 (HTLV-1) is of particular interest since its dissemination in vivo strictly depends on cell contacts. HTLV-1-infected T lymphocytes are predisposed to form “virological synapses”, but the detailed mechanism of HTLV-1 transmission from cell to cell remains poorly understood. Using T cells from naturally infected patients, we show that HTLV-1-infected T lymphocytes transiently store viral particles in extracellular assemblies that are held together and attached to the cell surface by virally-induced extracellular matrix and cellular linker proteins. Extracellular viral assemblies rapidly adhere to other cells upon cell contacts allowing virus spread. Their removal strongly impairs the capacity of HTLV-1-producing cells to infect target cells. Our data unveil a novel virus transmission mechanism based on the generation of extracellular viral particle assemblies whose structure, composition and functions resemble those of bacterial biofilms. HTLV-1 biofilm-like structures promote the infectious capacity of the virus enhancing its efficiency of transmission to other cells. These results open new perspectives for antiviral strategies.

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MUTANTS IN THE SIV CAPSID THAT AFFECT INFECTION EFFICIENCY

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Recently there have been discovered cytosolic proteins that block retroviral infection at an early post-entry stage or are required for steps in the pre-integration life cycle. The target of these proteins is likely the incoming viral core, specifically the capsid protein. Our data, and other work, support the possibility that core stability, and the appropriate host environment, are required for proper core processing, both temporally and spatially. We report here the discovery and initial study of SIV CA mutants whose phenotypes suggest a failure to meet these essential requirements. These mutants may prove to be useful tools in assessing CA function in early post-entry events.

PLATELETS INHIBIT HIV-1 SPREAD IN AN ACTIVATION-DEPENDENT MANNER

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A fraction of HIV-1 in the blood of infected patients is associated with platelets and platelets are often found in an activated state in HIV-1 infection. We could previously show that the C-type lectin DC-SIGN and the lectin-like molecule CLEC-2 are expressed on platelets and facilitate HIV-1 capture by these cell fragments. Here, we investigated how the activation status of platelets impacts the interaction with HIV-1, and we determined the consequences of the HIV-1 association with platelets for viral infectivity.

We show that platelet activation significantly increases the ability of these cell fragments to facilitate infection of adjacent C8166 T-cells, and this activity was dependent on DC-SIGN and CLEC-2, in agreement with our previous results. The HIV-1 transmission assay employed for these studies involved removal of unbound virus and was designed to model early steps in HIV-1 transmission. In order to assess the impact of platelets on infectivity of virions present in the blood of infected patients, we chose a closed culture system, in which T-cell-platelet cocultures were infected with HIV-1 without removal of unbound virus. Under these conditions, platelets reduced HIV-1 replication efficiently and in a concentration-dependent manner. Notably, the antiviral function of platelets was dependent on the platelet activation status, with resting platelets being significantly more adept to suppress HIV-1 spread than activated platelets. Experiments with a transwell tissue culture system revealed that the antiviral activity of platelets did not require contact with T cells, suggesting that platelets reduced viral infectivity by sequestering free virus and/or by secreting soluble factors. Indeed, the supernatants of activated but not resting platelets profoundly suppressed HIV-1 spread, indicating that resting platelets store antiviral factors which are released upon activation and block HIV-1 infection.

In summary, our results demonstrate a so far unappreciated dual role of platelets in HIV-1 infection: Upon introduction of HIV-1 in the blood stream by e.g. needle stick injury activated platelets might capture and transfer the virus to T cells. In contrast, in the context of chronic infection, constitutive activation of platelets might result in a constant release of antiviral factors. These results help to explain the previously reported association of platelet counts, viral load and disease progression, and highlight platelets as a component of the antiviral immune response.

DITHIOTHREITOL CAUSES HIV-1 INTEGRASE (IN) DIMER DISSOCIATION WHILE SMALL MOLECULES INTERACTING WITH THE IN DIMER INTERFACE PROMOTE DIMER FORMATION

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We have developed a homogeneous time-resolved FRET-based assay that detects the formation of HIV -1 integrase (IN) dimers. The assay utilizes IN monomers that express two different epitope tags that are recognized by their respective antibodies, coupled to either allophycocyanin or europium cryptate. Surprisingly, we find that dithiothreitol (DTT), a reducing agent essential for in vitro enzymatic activity of IN, weakens the interaction between IN monomers. This effect of DTT on IN is dependent on its thiol groups since the related chemical threitol which contains hydroxyls in place of thiols has no effect on IN dimer formation. However, by studying point and deletion mutants of IN, we determined that cysteine residues in IN seem to be dispensable for this dimer dissociation effect of DTT. Peptides that interact with the IN dimer interface were tested in our novel IN dimerization assay. These peptides displayed an intriguing equilibrium binding dose response curve characterized by a plateau rising to a peak, and then descending to a second plateau. Mathematical modeling of this binding system revealed that the shape of this dose response curve was consistent with the ability of these peptides to promote IN dimerization and block subunit exchange between IN dimers. This dose response behavior was further extended to tetraphenylarsonium chloride, a small molecule that interacts with the IN dimer interface. By analyzing the kinetics of IN heterodimer formation in the presence of the peptides and this small molecule, we determined their rate constants (i.e. k_{on} and k_{off}) and dissociation constant (K_d). In conclusion, our novel FRET-based IN dimerization assay has revealed that peptides and small molecules interacting with the HIV-1 IN dimer interface promote dimer formation and inhibit dimer subunit exchange.

A NOVEL POST-ENTRY INHIBITOR OF HIV-1 REPLICATION TARGETING THE CAPSID DOMAIN OF GAG

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Human immunodeficiency virus type 1 (HIV-1) Gag assembles into viral particles at the plasma membrane and, during the viral entry, the mature capsid (CA) dissociates from each other. We established an yeast two-hybrid system to measure the Gag-Gag interaction adopting the SOS signaling pathway. Using this system, we conducted a screening of Gag-Gag interaction inhibitors from a chemical library composed of 20,000 compounds, and isolated 6 candidates for Gag assembly inhibitors. When tested in human MT-4 cell line and primary peripheral blood mononuclear cells, one of the candidates (2-(benzothiazol-2-ylmethylthio)-4-methylpyrimidine, BMMP) displayed an inhibitory effect on HIV-1 replication in a dose-dependent manner, although a considerably high dose of the compound was required (5-20μM). The virus production and Gag processing were not significantly inhibited by BMMP. In contrast, single round infection assay with envelope-psuedotyped HIV-1 vector revealed that BMMP inhibited the postentry phase of HIV-1 life cycle. The compound did not block the postentry stage in SIV or MLV infection. Interestingly, experiments with HIV x SIV Gag chimeras indicated that CA was critical for BMMP-mediated HIV-1 block. The assembly of CA was inhibited in the presence of BMMP in vitro. Collectively, BMMP is an inhibitor of HIV-1 replication and the primary target of BMMP is the CA domain of Gag. As the chemical structure of BMMP is distinct from Gag inhibitors previously reported, BMMP may serve as a lead compound for the development of novel antiretroviral drugs.

DIFFERENCES IN PROVIRAL LATENCY OF HIV-1 SUBTYPES B AND AE

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The current HIV-1 pandemic is caused by at least nine subtypes (termed A through K) and an increasing number of recombinant forms. There has been a large skew in research focus on subtype B, which is the most prevalent subtype in the Western world. We study the transcriptional promoter in the long terminal repeat (LTR). Each subtype has a specific LTR promoter configuration and even minor changes in the transcription factor-binding sites (TFBS) or their rearrangement can have a significant impact on cell tropism and pathogenicity. For the HIV-1 subtypes, it is largely unknown to what degree LTR promoter variation contributes to differences in proviral latency. Such differences in latency properties may have an impact on the establishment of viral reservoirs and the inability to clear the virus by therapeutic intervention.
We have constructed recombinant viral genomes in which the subtype-specific promoters are cloned in the common backbone of the subtype B LAI isolate. The recombinant viruses are isogenic except for the core promoter region with all major TFBS (e.g. NF-kB and Sp1 sites). To measure proviral latency, we set up a single round infection system in the SupT1 T-cell line. The culture is split after 24 hours and either untreated (control) or treated with different compounds to activate latent proviruses. We measured the number of virus-producing cells (intracellular CA-p24 production) at 2 days post infection and determined the induction index (CA-p24 activated/CA-p24 control) for the different HIV-1 subtypes. Considerable proviral latency is apparent in this experimental model. A 3-fold increase in the percentage of virus-producing cells was triggered by TNFα for all subtypes, except AE. In subtype AE, one of the two regular NFκB sites is replaced by a GABP site. The GABP-to-NFκB mutation in the subtype AE promoter reduces basal transcription and restores the TNFα response. The reciprocal experiment, NFκB-to-GABP mutation in subtype B, does not increase basal transcription, although the TNFα response is reduced. Thus, the GABP site is not the sole determinant of these LTR properties and we are currently investigating other sequence variation between subtype AE and B to elucidate this difference.

ROLE OF UPSTREAM AUG CODONS WITHIN THE SIVMAC239 GENOME

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Simian immunodeficiency virus from Rhesus macaques (SIVmac) is a primate lentivirus that exhibits extensive similarities with human immunodeficiency viruses (HIVs) in morphology, genome organization and biological properties. Like HIV, SIV is dependent on the host cellular machinery for transcription, translation and protein production. Alternative RNA splicing generates many mRNAs that allow the expression of all viral proteins. Consistent with the idea that translation occurs predominantly via a cap-dependent scanning mechanism, one usually does not find AUGs upstream of the open reading frames (ORFs) in HIV and SIV.

In SIVmac239, the envelope (Env) glycoprotein is translated from a 4 kb mRNA which also contains the upstream ORF (uORF) for Rev. It is currently accepted that the level of Env expression is dependent on leaky scanning due to suboptimal translation initiation at the upstream Rev AUG. Interestingly, another potential start codon is present immediately upstream of the Rev-AUG. We also identified an alternative Rev-Env mRNA that has in fact four upstream AUGs, raising questions about the regulation of Rev and Env translation. We constructed subgenomic Rev-Env reporter mRNAs to test the contribution of the different upstream AUGs on protein expression. Some mutations were also introduced into the infectious SIVmac239 molecular clone. The results indicate that the virus requires a delicate balance of protein translation.

SAFE T-CELL LIAISONS AND VIRUS TRANSMISSION PROMOTED BY THE HTLV-1 P8 PROTEIN

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Human T-cell leukemia/lymphoma virus Type I (HTLV-1) causes a persistent infection in T-cells that can culminate with leukemia. HTLV-1 is transmitted through cell-to-cell contact upon the formation of the virological synapse. However, the nature of the intercellular connections in HTLV-1 transmission remains unknown. HTLV-1 encodes a 12kD (p12) protein that resides in the ER and is cleaved to remove an ER retention/retrieval signal to generate the p8 protein, which traffics to the cell surface. The p12 and p8 proteins exert opposite effects on T-cells. The p12 protein induces T-cell activation by increasing ER calcium influx and NFAT activity and T-cell proliferation, by binding the IL-2 receptor γ and β chains and increasing STAT-5 phosphorylation and IL-2 production. In contrast, p8 upon T-cell receptor (TCR) ligation is recruited to the immunological synapse (IS), the contact site between the antigen presenting cell (APC) and the T-lymphocytes, and down-regulates TCR signaling causing anergy. Here we reconcile this apparent paradox and found that the p8 increases T-cells contact and the number and length of Tunneling Nano-Tubes (TNT), cellular conduits that establish a physical communication network among dendritic cells and T-cells, both targets of HTLV-I infection. Strikingly, p8 is rapidly transferred to neighboring cells through direct cell-cell contacts and TNT, which may prime surrounding T-cells for viral acquisition. The increase of cell-cell contact is explained in part by the clustering of LFA-1 adhesion molecule on the cell surface. Quantitative analysis revealed HTLV-1 p8 significantly increases transmission of the HTLV-1 virus to neighboring cells. The core (Gag) and Envelope proteins of HTLV-I and p8 were visualized in the TNTs by confocal microscopy and live imaging. Electron microscopy demonstrated virus particles are associated with TNT formed following p8 expression. The ability of p8 to simultaneously induce T-cell anergy and cluster T-cells by TNT formation represents a novel and elegant example of virus adaptation in an immune competent host. This model identifies a novel viral target for intervention in HTLV-I infection.

DROSOPHILA MELANOGASTER AS AN IN VIVO MODEL FOR STUDYING ENVELOPE GLYCOPROTEINS OF MAMMALIAN RETROVIRUS: THE CASE OF JAAGSIEKTE SHEEP RETROVIRUS (JSRV).

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Betaretrovirus *Jaagsiekte Sheep Retrovirus* (JSRV) is the etiological agent of ovine pulmonary adenocarcinoma (OPA), a contagious lung cancer of sheep. It was previously shown that the expression of JSRV envelope protein (JSRVEnv) alone can induce transformation of epithelial cells *in vivo* and *ex vivo*. The transformation led to activation of several signaling pathways involved in cellular proliferation and growth but the precise interplay between JSRVEnv and these pathways are still unclear. *Drosophila* is a convenient model to investigate to growth, proliferation and cell death mechanisms at the genetic level, because most signaling pathways are well conserved between vertebrates and invertebrates. We have generated Drosophila transgenic lines. Inducible and tissue-specific expression of JSRVEnv were observed into eyes and wings tissues. Two interesting results were obtained: 1- the whole-wing size was significantly reduced when JSRVEnv was expressed in the tissue leading to the adult wing due to a reduction of cell size. 2- Apoptosis was induced in larval and adult eyes as shown by immunostaining. Interestingly, a recent study has shown that apoptosis could induced mechanism of compensatory proliferation. Therefore, JSRVEnv could use this mechanism to cause cancer. We are currently testing this hypothesis using our experimental model.

INTEGRATION SITE ANALYSIS IN XMRV-POSITIVE PROSTATE CANCERS

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Xenotropic MLV-related retrovirus (XMRV) is a novel human retrovirus discovered in prostate cancers. In our patient population, 27% of all prostate cancers tested XMRV-positive by immunohistochemistry (IHC) or quantitative PCR (qPCR); the prevalence rates vary in the different studies done to date. It is not certain whether the virus causes the prostate cancer. Other gammaretroviruses cause cancers by insertional activation of oncogenes by viral LTR sequences or by insertional inactivation of tumor suppressor genes. If XMRV causes cancers, it is likely to follow a similar mechanism.

To establish a causal role for XMRV in human cancers, we are looking to identify XMRV integration sites in human prostate cancers, and to determine their relationship with oncogenes and tumor suppressors. For this analysis, patient genomic DNA is digested with a restriction endonuclease and ligated to an adapter linker, which serves as a primer binding site. PCR amplification using a primer for this site and a second primer in the XMRV 3'LTR allows selective amplification of integrated XMRV LTRs with the adjoining host cell DNA. Tens of thousands of these PCR products can then be sequenced simultaneously by high-throughput 454 sequencing.

The fact that human cancers have very few copies of XMRV proviral DNA has posed a significant challenge for all scientists studying XMRV in patients. Methods to overcome these challenges will be discussed. Finding a set of integration sites that are close to an oncogene and clonal in a tumor would strongly suggest a causal role for XMRV in prostate cancer.

COMPARISON OF THE ASSEMBLY AND REPLICATION PROPERTIES OF pNL4-3 AND pHXB2R3 MOLECULAR CLONES AND SELECTION OF ENV AND VPU VARIANTS

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HIV-1 Gag expression is necessary and sufficient for the production of virus-like particles (VLPs) in mammalian cells. Gag is therefore the minimal viral determinant required for VLP assembly and release. Studies exploring HIV-1 Gag trafficking pathways have used different Gag expression vectors in different cell types, in some cases with contrasting results. Compared with a panel of commonly used HIV-1 Gag expression vectors in different cell lines (e.g., HeLa, and 293T), two X4-tropic proviral clones, pNL4-3 and pHXB2R3 (Vpu defective), showed distinct properties in virus release (HeLa, 293T, and Jurkat), infectivity (TZM-1b), and replication (Jurkat) assays. pNL4-3 shows higher release efficiency, higher infectivity, and faster replication kinetics than pHXB2R3. Not only Vpu and also additional as-yet-unknown factors contribute to the differences observed between these two proviral clones. HXB2R3 or HXB2R3U viruses collected at the peak of virus replication in Jurkat cells demonstrated accelerated replication capacity in the second round of infection. Mutations were identified in pHXB2R3/pHXB2R3U Vpu and/or Env: pHXB2R3 Env531AT and pHXB2R3U Vpu52SI/Env403AT or Env33YH. Replication defects were corrected in all clones by the Env mutations and improved to a lesser degree with the Vpu52SI mutation. Further molecular/cellular analyses will be presented to explore the basis for these effects.

INHIBITION OF HIV REPLICATION BY DOMINANT-NEGATIVE INTERFERENCE WITH WT VIF FUNCTION

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Members of the host restriction factors, apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) super family, mainly A3G and A3F are potent inhibitors of retroviruses and retroelements. In the absence of Vif, these APOBEC molecules are incorporated into budding virions to induce cytidine deamination on the minus stand viral DNA, inhibit cDNA synthesis, and inhibit integration of newly infected cells. HIV-1 Vif overcomes this host restriction by binding to the N-terminal region of APOBEC, and recruiting the E3-ubiquitin ligase machinery consisting of Cullin 5- EloginB/C to the C-terminal domain of Vif, thus polyubiquitylating and targeting APOBEC for proteasomal degradation. To identify dominant-negative (dn) effects of Vif mutants on APOBEC, we constructed non-functional Vif variants containing either mutations and/or deletions of key substrate binding domains to APOBEC, Cullin 5/Elogin B-C, and potential sites of Vif homodimerization. These Vif mutants with the potential to interfere with the function of wild type Vif were co-expressed with wild type HIV-1 NL4-3. Their ability to promote the packaging of A3G and A3F into budding virions and reduce the infectivity of HIV-1 in target cells was determined. Our earlier studies show that the expression of mutations of Vif at the C-terminus counteracted the Vif-induced reduction of intracellular A3G levels presumably by preventing Vif-induced A3G degradation. Consequently, dn Vif interfered with wild type Vif's ability to exclude A3G from viral particles, and reduced viral infectivity despite the presence of wild type Vif. Currently, we have expanded our studies to test the dn properties of these Vif mutants in the presence of A3F. Despite the fact that the domains in A3G/A3F and Vif required for interaction are distinct, we found that the dn properties of our Vif mutants applied to both A3G and A3F. We are currently testing our mutants in conjunction with other Vif-sensitive deaminases to see if dn interference by Vif is a general non-selective characteristic or restricted to certain deaminases. The ability of our dn Vif mutants to induce packaging of APOBEC in the presence of wt Vif offers a promising target for drug therapeutics in the treatment of HIV/AIDS.

K101E+G190S MUTATIONS OF HIV-1 REVERSE TRANSCRIPTASE (RT) CONFER STIMULATION OF VIRAL REPLICATION BY NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIS).

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Review of the Stanford drug resistance database for clinical occurrence of combinations of NNRTI resistant mutants shows that 37% of isolates with the mutation G190S in RT also had K101E. To determine whether the double mutant K101E+G190S has an advantage over G190S alone, we introduced K101E+G190S into an NL4-3 backbone and measured in PM1 T cells and the viral fitness using growth competition assays in the absence of efavirenz (EFV) and viral resistance in the presence EFV. Surprisingly, we found that the replication of NL4-3 (K101E+G190S) was stimulated in the presence of drug compared to the absence (2-fold maximum stimulation at 400nM EFV). NL4-3 (K101E+G190S) reduced the fitness (41%) and increased the resistance 47-fold compared to G190S alone. NRTI resistance mutation L74V improved the fitness of K101E+G190S 32% in the absence of drug, and abolished stimulation without affecting. M41L+T215Y improved the fitness of K101E+G190S 12% and abolished stimulation, but reduced the IC50 52-fold. To confirm the stimulation of K101E+G190S by drug, we cloned an RT with these mutations from a patient that failed EFV. This clone, D10, which has the resistance mutations M41L, K101E, G190S, and T215Y, showed 20-fold maximum stimulation at 800 nM EFV. Stimulation was eliminated when K101E was back-mutated, and resistance decreased as well (87-fold). In contrast, stimulation increased 6-fold and resistance increased 3-fold when M41L+T215Y were back-mutated. These results indicate that polymorphisms in the D10 clone can influence the level of stimulation compared to an NL4-3 backbone. These studies were confirmed in PBMCs. Stimulation was also observed with the NNRTI nevirapine (NVP), but not with etravirine. Studies of the mechanism of stimulation showed that EFV did not increase the specific polymerase activity or heterodimer formation of recombinant RT, nor did the drug increase gag-pol processing in the virion. These results show that some NNRTI mutations are stimulated by drug and that stimulation is eliminated by the addition of nRTI resistance mutations. When anti-retroviral treatment includes nRTIs and NNRTIs, stimulation may influence which mutations are selected in vivo. Newly developed NNRTIs, which do not stimulate NNRTI mutants, such as etravirine, would be preferred over others, such as EFV and NVP.

STUDY ON THE CELLULAR MECHANISM REGULATING HIV INFECTION OF MEMORY AND NAÏVE CD4 T CELLS.

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HIV-1 preferentially establishes latent infection in memory CD4 T cells, but the underlying virological and cellular mechanisms remained largely undefined. Here we studied the differential susceptibility of memory and naïve CD4 T cells to HIV infection, and found that memory CD4 T cells supported much higher levels of HIV-1 latent infection when compared with naïve CD4 T cells. However, when HIV was pseudotyped with the VSV-G glycoprotein, the virus is equally capable of infecting both memory and naïve T cells, either pre-activated or immediately activated after infection. These results suggested that the differential ability of HIV to infection memory and naïve T cells is at the early steps such as entry and/or early post entry events. We detected no differences on the surface levels of CD4 and CXCR4 between memory and naïve T cells, and, as a consequence, the fusion efficiencies are comparable in both cell subsets. However, we found that viral DNA synthesis and nuclear localization were much more efficient in memory T cells. The cellular mechanisms regulating these early viral processes are further investigated, and indeed we found distinctive cellular environment between these two cell subsets.

THE ROLE OF RNA HELICASES IN HIV-1 ASSEMBLY

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Central to the development of much needed new treatments for HIV-1 is a more thorough understanding of the viral lifecycle and the cellular co-factors required by the virus. This will allow the identification of novel drug targets. Targeting cellular proteins and their interaction with the virus is likely to reduce the problem of emerging viral resistance to drugs. We are particularly interested in cellular proteins which are required for the assembly phase of the viral lifecycle. During assembly, full length viral RNA fulfils dual roles. It serves as an mRNA to produce viral structural proteins and is packaged into assembling virions as genomic RNA. We hypothesise that the conformation of the viral RNA may be important in the spatiotemporal control of these two functions. Chaperone proteins, such as cellular RNA helicases, may be required for regulating the conformation of the viral RNA, and so the outcome of viral infection. To date, seven cellular RNA helicases have been proposed to play roles at various stages of the HIV-1 lifecycle.

With the aim of identifying further RNA helicases required for viral assembly we carried out a targeted siRNA library screen in HeLa cells. siRNAs targeting 59 cellular RNA helicases were tested for their effect on viral replication. Both viral protein production and infectious virion formation were assessed. Five RNA helicases were identified which reproducibly decreased viral replication when knocked down. The helicases identified are DDX5, DDX10, DDX17, DDX28 and DDX52. Whilst DDX5 and DDX17 have a variety of well established functions in RNA metabolism, the cellular functions of the other helicases are not known. We are currently carrying out overexpression and siRNA rescue experiments and will go on to characterise the interaction between HIV-1 and the RNA helicases identified in this siRNA screen.

HIV-1 VPR ENHANCES VIRAL REPLICATION IN DENDRITIC CELLS: MECHANISMS AND IMPLICATIONS

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HIV persistence is the major barrier to AIDS treatment using highly active antiretroviral therapy. Understanding the mechanisms of HIV persistence is essential for developing effective strategies to eradicate AIDS. The HIV-1 protein Vpr is a small viral accessory protein that contributes to HIV-1 replication and pathogenesis through dysregulation of a number of cellular events, including cell cycle, apoptosis, and host and viral gene expression. Vpr can also impair the immune functions and may play an essential role in HIV-1 immune evasion. Monocyte-lineage cells, including monocytes, dendritic cells (DCs) and macrophages, are critical for HIV-1 infection and transmission. Previous studies indicate that HIV-1 Vpr enhances HIV-1 replication in monocytes and macrophages in vitro, which likely contributes to persistent HIV-1 infection in vivo. Vpr specifically recruits the nuclear form of uracil DNA glycosylase (UNG2), a cellular DNA-repair enzyme, into HIV-1 virions. A Vpr mutant (W54R) HIV-1 that fails to recruit UNG2 into virions demonstrates a significant replication defect in macrophages. However, the role of Vpr in HIV-1 infection of DCs and in viral persistence remains unknown. We hypothesize that Vpr contributes to viral persistence by promoting HIV-1 infection of monocyte-lineage cells and impairing the immune functions of DCs. Our preliminary studies demonstrate that: (1) Vpr is required for efficient HIV-1 infection of monocyte-derived DCs; (2) Vpr complementation modestly enhances Vpr-defective HIV-1 replication in DCs; (3) Vpr enhances HIV-1 reverse transcription and nuclear import of viral cDNA in DCs; (4) A single mutation in Vpr (W54R) significantly impairs HIV-1 replication in DCs, but not in peripheral blood mononuclear cells. These results indicate that Vpr promotes HIV-1 replication in monocyte-lineage cells, suggesting a potential mechanism of HIV-1 persistence. We are investigating the mechanisms by which Vpr enhances HIV-1 infection in DCs. Our studies will provide important new information to understand the function of Vpr in HIV-1 pathogenesis.

MOLECULAR BASIS FOR NCP7'S DISTINCTIVE ROLE IN REVERSE TRANSCRIPTION

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The nucleic acid chaperone activity of HIV-1 NCp7 is crucial for efficient and specific reverse transcription. NCp7 precursors, NCp9 (NCp7-SP2), NCp15 (NCp9-p6), and Gag, are also nucleic acid chaperones. Yet, NCp9 and NCp15 do not have any known role in viral DNA synthesis, while Gag facilitates primer placement. To determine the molecular basis for these findings, we monitored chaperone function, using a sensitive (-) strand transfer assay that measures annealing of (-) strong-stop DNA to acceptor RNA and strand transfer (annealing plus DNA elongation). Assay of these activities revealed that NCp9 was only slightly less active than NCp7. However, NCp15's strand transfer activity was lower than that of the other two NCs and 4-fold more protein than NCp9 was required to reach a comparable rate and extent of annealing. This is not due to lower nucleic acid binding affinity, since the three NCs have similar *K*d values. Possibly, interaction of the acidic p6 domain with basic residues in the protein inhibits chaperone activity, as shown for HTLV-1 NC[1]. Gag had the greatest annealing activity and nucleic acid binding affinity, consistent with its role in primer placement, but its strand transfer activity was abolished at 0.46 μM, a concentration at which the NCs are active. This result reflects Gag's slow dissociation from bound nucleic acid (in contrast to NCp7's high on-off rate)[2] and suggests that Gag acts as a "roadblock" to DNA elongation by blocking RT movement along the template. Preliminary data show that NCp9 and NCp15 dissociate more rapidly than Gag, but slower than NCp7. Taken together, our results indicate why NCp7 has evolved as a critical cofactor in reverse transcription. This work was funded in part with federal funds from NCI, NIH, under contract HHSN261200800001E.

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RESISTANCE TO BROADLY NEUTRALIZING ANTIBODY DOES NOT ALTER REPLICATION FITNESS OF EIAV VARIANTS

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The development of broadly neutralizing antibody (NAb) is important for immunological control and clinical quiescence in EIAV-infected horses. Over time, however, viral genotypes evolve that resist NAb, resulting in recrudescence of clinical disease. We previously reported that variation in a 400 nt region of EIAV env, which contains the principle neutralizing domain (PND), conferred resistance to broadly neutralizing antibody. Chimeric viruses containing the PND1 genotype were readily neutralized by both the heterologous and autologous sera whereas chimeric viruses containing the PND5 genotype were neutralization resistant. Genetic changes in PND5 included size variation of V3 loop as well as increased numbers of potential N-link glycoslyation sites. These changes may facilitate immune evasion by potentially masking or shielding neutralizing epitopes, but are also thought to incur a cost in virus replication fitness. To address this question, we used direct growth competition experiments to compare the relative fitness of PND1 and PND5 virus. Equine dermal cells were infected with 100 FFU of PND1 and/or PND5 at an MOI of 0.002. At sequential times, supernatant and cells were collected, and the viral RNA and proviral DNA were quantified using a highly sensitive, PND type-specific quantitative real-time PCR assay. The results showed that both viruses spread through the cultures with similar kinetics; the number of PND1- and PND5-infected cells increased at a similar rate from day 1 to day 6 and reached maximal levels at day 9. Virion production, as measured by supernatant copy number, was also similar between PND1 and PND5, and the two viruses did not different in overall estimates of relative fitness. To further investigate the infectivity of PND1 and PND5 virus, we used pseudotype reporter viruses containing different reporter genes in single round infectivity assays. Preliminary results indicate no differences in infectivity of PND1 and PND5 pseudovirus. Together these results suggest that NAb–driven changes in EIAV PND region that confer resistance to broadly neutralizing antibody are not accompanied by a loss of replication fitness.

DIRECT FUNCTIONAL ANALYSIS OF VPX PROTEINS IN HUMAN MACROPHAGES

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Primary lentiviruses contain accessory genes that are essential for infection and spread. Simian immunodeficiency viruses (SIV) and HIV-2 but not HIV-1 contain Vpx which was the result of gene duplication of an ancestral Vpr. We and others have shown that Vpx is indispensable for SIV and HIV-2 infection of primary macrophage and dendritic cells. In addition, we found that the restriction was active against HIV-1 and Vpx enhanced HIV-1 infection of macrophage. We demonstrated that Vpx supports virus infection by counteracting a cellular restriction that is expressed in myeloid cells. This function of Vpx is largely depending on either a functional proteasome, or on its binding partners VprBP and DDB1 in target cells. Several Vpx mutants, which lose either the ability to form a complex with VprBP and DDB1, or the ability to be ubiquitinated, have been described as having a functional defect. Previous studies have been performed on either packaging Vpx variants in trans into SIV viral particles, or producing SIV viruses harboring wild type of mutant Vpx alleles. However, the efficiency of packaging of Vpx and its mutants varied significantly, which impaired the functional analysis of Vpx in target macrophages. Furthermore, the ability of SIV and HIV-2 to infect human macrophages can impact infection efficiency. It is therefore difficult to compare the requirement of Vpxs in target cells from different species.
In this study, we used a HIV-1 based lentiviral vector (HIVec2GFP), in which we cloned SIVpBj Vpx, its derived VprBP and DDB1 mutants, ubiquitination defective lysine mutants, as well as HIV-2 ROD Vpx and AgmVer Vpx in the place of GFP. HIVec2GFP efficiently infected primary macrophages from a variety of donors. We evaluated the ability of this vector to deliver Vpx to macrophages and to rescue the infectivity defect of a Vpx-defective virus in macrophages. We examined the stability of different Vpxs proteins in macrophages and correlated that with their ability to rescue a Vpx virus or to enhance HIV-1 infectivity. We found that ROD Vpx enhanced HIV-1 infectivity better than SIV pBj, while pBj Vpx more effectively rescued SIV. Agm Vpx failed to rescue HIV-1, HIV-2 or SIV Both ubiquitination mutants and VprBp-DDB1 binding mutants were functionally defective. However, we did not find a correlation between the amount of Vpx protein that was expressed and its ability to rescue in macrophages. ROD Vpx is the most stable protein in macrophage, while pBjVpx was the least. Furthermore, the turnover of Vpx protein was accelerated in macrophage as compared to cell lines.

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PRESENTING HIV-1 ENTRY INHIBITORS ON THE SURFACE OF LACTIC ACID BACTERIA TO CAPTURE AND INACTIVATE VIRAL PARTICLES

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Lactic acid bacteria (LAB) are an important part of our body microbiota, which naturally inhabit the human genital tract (e.g. vaginal) and gastrointestinal (GI) tract (e.g. rectum) in a large quantity. We are trying to employ these bacteria for the prevention of sexual transmission of human immunodeficiency virus (HIV-1).

A specific shuttle construct has been used to express anti-HIV inhibitors on the surface of LAB. The inhibitors include soluble CD4, monoclonal antibodies (e.g. b12) and the T20 peptide. The expression of these inhibitors has been detected and verified by several approaches such as Western blotting, immunofluorescent microscopy and flow cytometry. The engineered LAB have demonstrated the ability to absorb and neutralize several different HIV-1 viruses, including difficult-to-neutralize HIV-1 primary viruses. The results indicate the potential for using bacteria that capture and inactivate the incoming HIV particles for the prevention of HIV-1 infection. As these bacteria naturally colonize the human body, this approach might become a potential long-term prevention strategy for HIV/AIDS sexual transmission.

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BIOPHYSICAL STUDIES OF THE CONFORMATION OF TETHERIN

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Tetherin/BST2 is a type II membrane glycoprotein, which has been shown as a potent restriction factor for multiple enveloped viruses. It has a unique topology with a short cytoplasmic tail, followed by a transmembrane helix, an extracellular domain (ED), and a glycosyl-phosphatidylinositol (GPI) anchor. Three cysteine residues in tetherin ED have been reported to be functionally important and contribute to dimerization of the molecule. We have purified wild type and cysteine mutants of recombinant tetherin ED and used size exclusion chromatography, light scattering, and cross-linking to study its native oligomerization state and the contribution of the cysteine residues. Our study demonstrates that tetherin ED exists as a stable dimer even without the formation of any disulfide bond, suggesting the cysteine residues might be involved in the formation of a higher oligomer state functionally important. We further used static light scattering and diffraction methods to study the conformation of tetherin ED. Out results confirm that tetherin ED exists as a coiled-coil and provide inside into its geometry.

XPR1 IS NECESSARY BUT NOT SUFFICIENT FOR XMRV ENTRY

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Xenotropic viruses are gammaretroviruses that can infect a wide variety of cells from heterologous species. Gibbon ape leukemia virus (GALV) and the recently identified human xenotropic murine leukemia virus-related virus (XMRV) represent examples of xenotropic viruses isolated from primates. Most cells resistant to infection by GALV or XMRV can be rendered susceptible after expressing its human receptor, PiT1 or Xpr1, respectively. We have now discovered that BHK hamster kidney cells retain their resistance to these viruses even when their respective receptors are expressed.

We determined that BHKPiT1 and BHKXpr1 resistance to infection mapped to the viral envelope protein implying that viral envelope-receptor interactions compromised viral entry. Resistance of BHKPiT1 to GALV infection is not due to reduced receptor level, since we demonstrated that PiT1 receptor levels on BHKPiT1 cells were comparable to susceptible cells. Furthermore, we provide evidence that, unlike resistance of BHK to certain murine retroviruses caused by cell specific N-linked glycosylation of respective receptors, failure of the human PiT1 to function as a receptor for GALV in BHK cells is not the consequence of cell specific glycosylation of human PiT1.

To further explore the mechanism of resistance of BHKPiT1 to GALV infection, we determined the effect of fusing BHKPiT1 and murine Mus dunni tail fibroblast MDTF cells. MDTF cells are resistant to GALV but can be rendered susceptible to infection after expression of human PiT1. We found that BHKPiT1 fused to MDTF cells are susceptible to infection by GALV raising the possibility that MDTF cells contain an ancillary factor absent in BHK cells that is required for efficient entry. These results indicate that both the ancillary factor and human PiT1 are required to facilitate efficient viral entry into BHK cells.

We are currently investigating whether the mechanism for BHKXpr1 cells resistant to XMRV infection is comparable to the resistance of BHKPiT1 to GALV. Using similar analyses, we will fuse BHKXpr1 with NIH3T3 cells and determine the susceptibility of the hybrid cells to XMRV. Our finding will provide important information as to whether an auxiliary factor is required for XMRV infection in cells expressing xpr1.

MODULATION OF HIV-1 INFECTION AT LATE PHASE BY AN INTEGRASE-INTERACTOR, HUWE1

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Integration, an indispensable step for retrovirus replication, is catalyzed by integrase protein (IN). Although a number of studies have identified cellular interactors to IN as co-factors for the integration function, some interactors appear to have distinct roles in several steps of virus replication other than integration. These pleiotropic effects of IN have been found from a series of extensive investigation on IN-mutant viruses. In this study, we show that Huwe1, a HECT-domain containing E3 ubiquitin ligase, is new cellular interactor for both Moloney murine leukemia virus (MoMLV) and human immunodeficiency virus type 1 (HIV-1) IN. The interaction was mediated through catalytic core domain and insensitive to nuclease treatment. In addition, Huwe1 was co-immunoprecipitated with MoMLV PICs in virus-infected cells, indicating that Huwe1 is incorporated into integration complex after viral entry. Huwe1 is known to catalyze poly-ubiquitination of Mcl-1 and p53 and induce consequent degradation of these proteins. Nevertheless, we did not observe Huwe1-dependent ubiquitination of HIV-1 IN in ectopic HIV-1 IN expressing cells. Also, we detected equivalent level of luciferase activity in Huwe1-knockdown and control cells after single-round luciferase-expressing HIV-1 infection. These data suggest that Huwe1 is an inert adaptor protein at early phase of the infection. On the other hand, depletion of Huwe1 in a human T cell line (MT-4 cells) resulted in increasing infectivity of HIV-1 virion released from virus-infected cells. Further IP-western blotting analysis revealed that Huwe1 interacts with HIV-1 Gag-Pol precursor protein through an IN region. These results suggest that Huwe1 somehow modulates efficient production of infectious virions in late events of the retroviral replication cycle.

COMPLEX CELL CYCLE ABNORMALITIES CAUSED BY HTLV-1 TAX

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HTLV-I is the causative agent of adult T-cell leukemia/lymphoma (ATL), a malignancy of $CD4^+$ T cells whose etiology is thought to be associated with the viral trans-activator, Tax. We have shown recently that Tax can drastically up-regulate the expression of $p27^{Kip1}$ and $p21^{CIP1/WAF1}$ through protein stabilization and mRNA trans-activation and stabilization respectively. The Tax-induced surge in $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ begins in S phase and leads to aberrant cell cycle progression that ends in cellular senescence. Importantly, HeLa and SupT1 T cells infected by HTLV-1 also arrest in senescence, raising questions about the notion that HTLV-1 induces proliferation of infected cells. Here we show that Tax causes the DNA replication licensing factor, Cdt1, to accumulate in S phase. The increased Cdt1 level in Tax-expressing cells correlated with DNA hyper-replication. Most Tax-expressing HeLa cells had dramatically elevated levels of $p27^{Kip1}$ and $p21^{CIP1/WAF1}$ as well as reduced levels of cyclin B1 and Skp2. They developed elongated S/G2, and were slow to progress into mitosis. The use of HeLa cell lines that express EGFP under the control of 18 copies of the Tax-responsive 21-bp repeat element (HeLa/18x21-EGFP) and fluorescent ubiquitin cell cycle indicators (HeLa-FUCCI) respectively in time-lapse microscopy further revealed that many Tax-expressing cells eventually progressed through S/G2, and entered into irreversible G1 arrest/senescence, with some bypassing mitosis altogether. Indeed, cells that failed mitosis were enlarged with exaggerated nuclei and expressed senescence-associated β-galactosidase, consistent with the development of mitotic defects and senescence. Interestingly, a small population of HeLa/18x21-EGFP cells was found to be capable of progressing into mitosis with high levels of Tax expression, suggesting that genetic or epigenetic changes can occur in cell culture to prevent Tax-induced senescence.

ANTI-TETHERIN ACTIVITIES IN NON-PANDEMIC HIV-1

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Phylogenetic studies indicate that HIV-1 was transmitted to humans from primates through four independent events, giving rise to groups M and N that are most closely related to SIVcpz, and groups O and P that are more like SIVgor. Only group M viruses have resulted in widespread human infections and are responsible for the worldwide AIDS pandemic. Group O infections are restricted to West and Central Africa and account for only a small percent of HIV-1 infections, Group N infections are even more rare and geographically restricted, and HIV-1 group P virus was recently identified in a single Cameroonian woman. Transmission of viruses from primate hosts would require the evolution of strategies to counteract human versions of anti-viral restriction factors such as TRIM5α, APOBEC and tetherin. It is possible that the lower penetration of the non-pandemic viruses into the human population reflects less than optimal adaptation to one or more of these factors.

Tetherin (BST-2/CD317/HM1.24) is an interferon-inducible restriction factor that inhibits the release of enveloped viruses from the plasma membrane. Different primate lentiviruses have evolved host-specific anti-tetherin activities as functions of Vpu (HIV-1, SIVmus/mon/gsn/den), Env (HIV-2, SIVtan) and Nef (SIVmac/sm/agm). Last year we reported that although the Vpu proteins from both group M and N strains are active against human tetherin, the group O Vpu has no such activity. Further investigations have now shown that group O Nef, Vpr and Env proteins do not counteract human tetherin, although the HIV-1 O Nef protein retains activity against primate tetherins (chimpanzee and macaque). In addition, a proviral clone (pCMO2.5) of HIV-1 O is sensitive to human tetherin restriction despite intact open-reading frames for all 9 HIV-1 genes. Together, this suggests that the lower rate of infection by HIV-1 group O isolates could arise, in part, from their inability to counteract human tetherin.

We have also investigated the basis for the lack of adaptation of HIV-1 O Vpu to human tetherin. Using chimeras of HIV-1 M and O Vpu, we mapped this difference to a region in the TM domain of Vpu that could represent a protein interacting domain. Confocal studies also suggest that the lack of activity of HIV-1 O Vpu could result from the different cellular localization of this protein compared to the functional group M and N Vpu proteins.

THE HOST PROTEINS XPB/XPD PARTICIPATE IN DEGRADATION OF RETROVIRAL CDNA IN THE NUCLEUS.

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The host helicases XPB and XPD restrict transposition of the LTR retrotransposon Ty1 and retroviral infection. Mutations of these essential proteins allow greater accumulation of retroelement cDNA. Retroviral infection was compared between cell lines derived from patients expressing XPB(F99S) or XPD(R683W) mutations and these cell lines complemented with the wild type genes. As part of the TFIIH complex, XPB and XPD are required for transcription. Inhibition of transcription by α-amanitin or translation by cycloheximide did not affect HIV cDNA accumulation in any of the XP cell lines, suggesting that XPB/XPD effects on cDNA are not via the transcription of secondary proteins. Previous results indicate that XPB/XPD participate in degradation of the retroviral cDNA. The host protein APOBEC3G may also degrade retroviral cDNA. Infection of the XP cell lines with HIV produced in cells expressing African green monkey APOBEC3G or human APOBEC3G suggest that the XPB/XPD pathway is distinct from the APOBEC3G degradation pathway. By arresting cells with aphidicolin, XPB/XPD proteins are exclusively nuclear. Retroviral cDNA accumulation was greater in arrested cells than in cycling cells. HIV cDNA accumulation in arrested wild type and XPB(F99S) cells is equal when the cDNA is in the cytoplasm, but is less in wild type cells when the cDNA has migrated to the nucleus. Similarly, MMLV cDNA accumulation is equal in arrested wild type and XPB(F99S) cells during aphidicolin mediated arrest. Since MMLV cannot enter the nucleus while the cells are arrested, the MMLV cDNA accumulates and remains stable until aphidicolin is removed. Following removal of aphidicolin, the MMLV cDNA is reduced. The rate of disappearance of MMLV cDNA is greater in wild type cells compared to XPB(F99S) cells. XPB/XPD mediated restriction of retroviruses appears to act through retroviral cDNA degradation in the nuclear compartment.

CONSTITUTIVE ACTIVATION OF STAT1 CAUSES SPONTANEOUS APOBEC3G EXPRESSION, WHICH DETERMINES PERMISSIVE PHENOTYPE AGAINST VIF-DEFICIENT HIV-1 REPLICATION IN T-CELL LINES

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HIV requires Vif to replicate efficiently in primary cells and certain T-cell lines (*e.g.*, H9, CEMx174, and MT-2), which are designated as "nonpermissive cells". In contrast, Vif is dispensable for replication in other cells (*e.g.*, SupT1, Jurkat, and Molt4), known as "permissive cells". APOBEC3G (A3G) was identified as the cellular factor. However, the details regarding cell-line dependent expression of A3G remain unknown. To address this issue, we analyzed regulated A3G expression in primary cells and compared the factors involved in the signal cascade of the cell lines.

Initially, we investigated A3G induction in primary macrophages (MDMs). Analysis of APOBEC3 mRNAs by quantitative real-time PCR revealed that A3G mRNA is significantly induced by interferon (IFN)α (18-fold), IFNβ (10-fold), or Toll-like receptor (TLR) 3 ligand (>20-fold), as previously reported. In contrast, IFNγ, or other TLR ligands marginally increase the A3G mRNA level in MDM. There are two types of signal transducers that commonly play key roles in the downstream cascade of IFNα, β, and TLR3 ligand stimulation: IFN-responsive transcription factors (IRFs), IRF-3, -7, -9 and STAT1 and 2. Therefore, we tested to see whether different expression patterns of these factors occur in permissive and nonpermissive cell lines. When the relative mRNA of IRF-3, -7, -9 and STAT1 among cell lines (H9, CEMx174, MT-2, A3.01, Molt4, Molt3, SupT1, Jurkat, U937, and THP-1) were measured by real-time PCR, A3G mRNA expression level in each cell was correlated with the STAT1 mRNA level. In addition, analysis by Western blot showed that the nonpermissive cell lines constitutively express STAT1 as well as phosphorylated STAT1 (activated forms phosphorylated at Tyr701 and Ser727), whereas the permissive cells commonly express significantly less. Moreover, treatment of H9 cells with an inhibitor that blocks the Janus kinase 1 (JAK1) for STAT1 activation decreased A3G expression, suggesting that JAK1 inhibitor enables the nonpermissive cell, H9 to exhibit a permissive cell phenotype. These results suggest that spontaneous A3G expression in T-cells, which is dependent on constitutive expression and activation of STAT1, determines whether *vif*-deficient HIV is capable of replication.

EFFECTS OF INTERFERON REGULATED PROTEINS, RNASE L AND APOBEC3G, ON XMRV REPLICATION

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XMRV is a human gammaretrovirus originally identified in a subset of prostate cancer patients with a subtle deficiency in the interferon (IFN) regulated antiviral protein, RNase L. IFN induces OAS proteins that produce 2'-5'-oligo(rA) activators of RNase L in response to viral dsRNA. Virus-host interaction studies could provide insight on the pathogenic consequences of XMRV infections as well as a means for controlling infections. Here we studied effects of candidate IFN regulated proteins on XMRV infections in prostate cancer cells and other cell types. Viral RNA levels were determined in Northern blots, protein levels in Western blots and viral release by RT activity in conditioned media. XMRV expression and replication was sensitive to IFN-beta treatment in both acutely- and chronically-infected DU145 cells. IFN-beta treatment decreased levels of XMRV genomic RNA while also preventing accumulation of viral proteins. Effects of RNase L were determined by decreasing its expression with RNA silencing. Cells with reduced levels of RNase L were partially resistant to the antiviral effect of IFN-beta. Moreover, after transfection with XMRV cDNA, RNase L null mouse embryonic fibroblasts (MEF) were less sensitive than wild type MEF to IFN inhibition of Gag expression. Apobec3G is a host restriction factor that suppresses retroviral infections and whose expression can be enhanced by type I IFN in a cell-type dependent manner. A comparison between prostate tumor cell lines and primary prostate cells (stromal and epithelium) suggested loss of apobec3G expression occurred during tumorigenesis. Expression of Gag protein as well as intracellular viral RT activity was lower in XMRV infected HEK/A3G cells than the parental HEK cells which lack apobec3G expression. No expression of apobec3G was observed in two prostate cancer cell lines, DU145 and LNCaP, while low levels of apobec3G were found in PC3 cells. In addition, IFN-beta did not induce apobec3G in these three cell lines. In contrast, tetherin/BST-2, was IFN inducible in DU145 cells. Interestingly, apobec3G was observed in primary prostate epithelial (PrEC) and stromal (PrSC) cells. These findings suggest that apobec3G is not responsible for the IFN anti-XMRV effect in DU145 cells, but tetherin and RNase L may still play a role. IFN induces many other antiviral genes, some of which are likely to be involved in the antiviral effect of IFN against XMRV. Studies in progress are aimed at identifying and studying which of these IFN induced proteins are inhibitory for XMRV infections.

SINGLE POINT MUTATION AT POSITION 140 IMPAIRS APOBEC3H PACKAGING INTO THE HIV-1 VIRION

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Four major A3H variants were reported to circulate the human population (HapI:18R/105G/121K/178E; HapII: 18R/105R/121D/178D, HapIII: d15N18R/105R/121D/E178D, HapIV:d15N18L105R/121D/178D). HapII, differing only in three amino acids from A3H-HapI, has the strongest inhibitory effect on both HIV-1 and Line-1. When introduced in vitro into cells, HapII has stronger protein stability in comparison to HapI and thus expressed at higher level. When expressed at similar expression level, both HapI and HapII can be packaged into the ΔVif HIV-1 virion in comparable level and have similar anti-HIV-1 activity.

7SL RNA is a host RNA that is selectively packaged into the HIV-1 virion and many other retroviruses. It plays important roles in mediating APOBEC3G (A3G) and APOBEC3F (A3F) packaging into HIV-1 virion. A unique motif RLLYF/YW is conserved on amino-terminal of A3G and A3F and is required for their binding of 7SL RNA and virion incorporation. However A3G and A3F still differ greatly in their ability to bind to 7SL RNA, indicating that other motifs maybe also invovled in 7SL RNA binding.

Interestingly, we found that the RLLYF/YW motif is conserved on all A3H variants. Furthermore, introduction of a single point mutation from 140E to K on A3H_HapI abolished its anti-HIV-1 activity even when expressed at high expression level. A3H_HapI_140K also lost its ability to associate with 7SL RNA and cannot be packaged into the ΔVif HIV-1 virion. Similarly, introduction of 140K to A3H_HapI_105R, A3H_HapI_105R/121E or A3H_HapII also greatly impaired their anti-HIV-1 activity. Therefore, we have newly identified amino acid 140E as a new motif important for A3H variants packaging into the HIV-1 virion.

IDENTIFICATION OF NOVEL VIF MOTIFS FROM HIV-1 THAT REGULATE APOBEC3G AND APOBEC3F NEUTRALIZING ACTIVITY

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Vif binding to APOBEC3G (A3G) and APOBEC3F (A3F) is a key step for A3G/A3F neutralization, and this mechanism has been extensively studied. So far, six discontinuous surfaces on Vif have been found to regulate A3G and/or A3F degradation. The 40YRHHY44 domain specifically binds to A3G and determines Vif specificity to A3G; the 11WxxDRMR17 and 74TGERxW79 domains specifically bind to A3F and determine Vif specificity to A3F; and the 21WxSLVK26, 55VxIPLx4L64, and 69YxxL72 domains determine Vif specificity to both A3G and A3F. Based on thse findings, it has been thought that Vif interacts with A3G and A3F mainly via its N-terminal region, and with Cul5 E3 ubiquitin ligase machinery via its C-terminal region.

Here, we identified two additional Vif regulatory motifs: one from the central region, and the other from the C-terminal region. Via sequence analysis of more than 2,000 different HIV-1 Vif proteins, we identified two highly conserved amino acid sequences 81LGxGxSIEW89 and 171EDRWN175. Within the 81LGxGxSIEW89 sequence, residues L81, G82, G84, and to a lesser extend I87 and W89, play very critical roles in A3G/A3F neutralization. Interestingly, residues L81 and G82 only regulate Vif activity to A3F, whereas residues G84, I87, and W89 regulate Vif activity to both A3G and A3F. Accordingly, this 81LGxGxSIEW89 sequence is designated as 81LGxGxxIxW89 domain. Within the 171EDRWN175 sequence, all residues except N175 are almost equally important for regulation of A3F neutralization. Accordingly, this domain is designated as 171EDRW174. Our binding assays indicated that the 81LGxGxxIEW89 domain determines Vif binding to both A3G and A3F, where the 171EDRW174 domain only determines Vif binding to A3F. The LGxGxxIxW domain is also partially conserved in SIVmac239, and has a similar activity. Our results indicate that A3G and A3F interaction surfaces on HIV-1 Vif are structurally complex, and more efforts are required for a complete understanding of this host-pathogen interactive mechanism.

HTLV-1 TAX CAUSES CELLULAR SENESCENCE BY DISRUPTING THE NF-κB/I-κBα AUTOREGULATORY LOOP

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Human T lymphotropic virus type 1 (HTLV-1) trans-activator, Tax, alters a multitude of basic cellular processes, including gene transcription, I-κB kinases (IKKs) signaling, cell cycle entry, DNA replication, and mitosis. These activities of Tax have been implicated in leukemogenesis. Expression of Tax in most cells, however, leads to drastic up-regulation of cyclin-dependent kinase inhibitors, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, and irreversible cell cycle arrest/senescence. Here we show that Tax causes senescence by disrupting the NF-κB/I-κBα auto-regulatory loop. Tax-induced senescence can be attenuated by amino acid substitutions in Tax that reduce IKK/NF-κB activation, and prevented by blocking NF-κB using a degradation-resistant mutant of I-κBα despite constitutive IKK activation by Tax. Small hairpin RNA-mediated knockdown of RelA, but not RelB, c-Rel or p100, prevents senescence induction by Tax. These results link unchecked NF-κB activation to Tax-induced cell cycle abnormalities and cellular senescence. We suggest that during viral infection, moderation of NF-κB activation by HBZ — a newly discovered HTLV-1 protein that negatively regulates RelA — may prevent Tax-induced senescence to allow proliferation of infected T cells, a pre-requisite for leukemia development.

FACTORS INFLUENCING THE RELATIVE CONTRIBUTIONS OF CELL-FREE AND CELL-ASSOCIATED HIV TO VIRAL SPREADING IN MODEL TISSUE CULTURES

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HIV can spread by either of two modes, via the cell-free aqueous environment or by direct cell-cell contact. The contributions of both pathways to HIV spreading are poorly understood and evidence for either pathway has been presented. Here we identify parameters that influence the relative contributions of both modes in a simple co-culture model system of HIV producing HEK293 cells and various T cells. Using this system, we demonstrate that experimental conditions that hinder cell-free virus spreading promote cell-to-cell spread. For instance, T lymphocytes that are poorly susceptible to cell-free HIV are readily infected when co-cultured with producer cells. These effects are possibly due to the high concentration of receptors at sites of cell-cell contact that facilitates virus entry and induce signaling cascades that activate target cells to promote subsequent HIV replication. Moreover, if viruses are retained at the surface of producer cells, release of cell-free virus is blocked, but HIV can still spread by cell-cell contact. Last, spreading from cells with low viral gene expression can be stimulated by co-culture with target cells, probably due to the enrichment of viral proteins at sites of cell-cell contact. Thus, we demonstrate that HIV favors cell-to-cell transmission under condition of low HIV gene expression and surface retention in the producer cell as well as poor susceptibility of target lymphocytes. Our data are consistent with the emerging model that several steps of the viral replication cycle can be efficiently coordinated at sites of cell-cell contact.

MECHANISMS OF HIV-1 RESTRICTION IN THE HUMAN T CELL LINE CEM.NKR

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Retroviruses are susceptible to a variety of host-derived restriction factors, but they have developed antagonistic mechanisms for productive infection of their hosts. Previously, we demonstrated that the CEM-derived cell line CEM.NKR expresses a factor that potently restricts the wild-type HIV-1 replication at a post-entry step. This factor is distinguishable from TRIM5α or any APOBEC3 cytidine deaminases. Here, we further characterized this unknown factor by testing whether it also targets several other retroviruses including amphotropic murine leukemia virus (MLV) and SIVs isolated from African green monkeys (SIVagm9063, SIVagmTan, SIVagmSab) and rhesus monkeys (SIVmac239). Although MLV replication was not, the replication of all tested SIV strains was effectively inhibited by this factor-mediated restriction in CEM.NKR cells. In addition, although this factor is constitutively expressed in CEM.NKR cells, it does not seem to be an interferon-inducible factor. Thus, CEM.NKR cells express a general antiviral factor that specifically targets primate lentiviruses. Since viruses have not yet developed a counteractive mechanism, further studies on this host factor may provide a new system to study viral evolution and open new avenues for antiretroviral therapies. Currently, we are mapping the major target for this factor on HIV-1, and we are also trying to rescue HIV-1 replication in CEM.NKR by small chemical compounds to understand this novel antiretroviral mechanism. These new progresses will be presented in this meeting.

TETHERIN RESTRICTS HIV-1 CELL-TO-CELL TRANSFER

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The IFN-inducible antiviral protein tetherin (or BST-2/CD317/HM1.24) impairs release of mature HIV-1 particles from infected cells. HIV-1 Vpu antagonizes the effect of tetherin. The fate of virions trapped at the cell surface remains poorly understood. Here, we asked whether tetherin impairs HIV cell-to-cell transmission, a major means of viral spread. Tetherin-positive or -negative cells, infected with wild-type or delta-Vpu HIV, were used as donor cells and cocultivated with target lymphocytes. We show that tetherin inhibits productive cell-to-cell transmission of delta-Vpu to targets and impairs that of WT HIV. Tetherin accumulates with Gag at the contact zone between infected and target cells, but does not prevent the formation of virological synapses. In the presence of Tetherin, viruses are then mostly transferred to targets as abnormally large patches. These viral aggregates do not efficiently promote infection after transfer, because they accumulate at the surface of target cells and are impaired in their fusion capacities. Tetherin, by imprinting virions in donor cells, is the first example of a surface restriction factor limiting viral cell-to-cell spread.

CELL-CELL SPREAD OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 OVERCOMES TETHERIN/BST-2 MEDIATED RESTRICTION IN T CELLS.

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Direct cell-to-cell spread of human immunodeficiency virus type-1 (HIV-1) between CD4+ T cells at the virological synapse (VS) is an efficient mechanism of viral dissemination. Tetherin (BST-2 /CD317) is an interferon-induced, anti-retroviral restriction factor that inhibits nascent cell-free particle release and is antagonized by the HIV-1 accessory protein Vpu, but whether tetherin can restrict cell-cell spread of HIV-1 is unknown. Here we show that Vpu-defective virus disseminates more efficiently than wild-type virus by direct T cell-T cell transmission because of increased VS formation. Tetherin, but not Vpu, was enriched at the T cell VS and colocalized with Env on infected T cells. Upregulating tetherin expression with interferon did not impair HIV-1 spread at the VS; instead interferon treatment increased VS formation. Moreover, siRNA knockdown of endogenous tetherin in Jurkat T cells and primary CD4+ T cells decreased, not enhanced, cell-cell transmission of Vpu-defective virus. We conclude that cell-cell spread of HIV-1 at the T cell VS can overcome tetherin-mediated restriction. In addition, tetherin may contribute to more efficient T cell-T cell transmission of Vpu-defective HIV-1. Since tetherin antagonism is conserved in primate immunodeficiency viruses, this suggest that one role of Vpu is to maintain a balance between cell free and cell-cell spread in the face to an innate immune response.

DISPLACEMENT OF TETHERIN FROM SITE OF HIV-1 ASSEMBLY AT THE PLASMA MEMBRANE BY VPU

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Tetherin directly blocks the release of nascent retrovirus particles from infected cells and is antagonized by Vpu. Vpu phosphorylation of its two cytoplasmic serines is required to recruit the ß-TrCP ubiquitin ligase and to induce cell surface downregulation and degradation of Tetherin. However, a Vpu mutant (Vpu2/6) that lacks these phosphorylation sites remains partially active as a tetherin antagonist. How this degradation/downregulation-independent activity of Vpu is manifested is unknown.

Here, we used light microscopy to demonstrate that Vpu2/6 was incapable of downregulating tetherin from the cell surface but was capable of displacing tetherin from nascent virions at the cell surface. A tetherin TM domain mutant that is resistant to antagonism by Vpu was resistant to this displacement activity. By constructing artificially membrane anchored Vpu proteins lacking the TM domain and phosphorylation sites that were directed to 'interact' with tetherin by direct fusion of the C-terminus of Vpu to the N-terminus of tetherin, we could demonstrate that the cytoplamic domain of Vpu, particularly the second predicted helical domain, was sufficient to delocalize tetherin from sites of viral assembly. Moreover, the artificially anchored, tetherin-fused Vpu antagonized the ability of tetherin to block particle release.

Previously, we showed that removal of the tetherin glycophosphatidylinisotol (GPI) anchor rendered it inactive as a viral tether. However, this tetherin(ΔGPI) mutant is efficiently incorporated into extracellular virions, in a manner that is blocked by Vpu. We used tetherin(ΔGPI)-based proteins to show that the artificially anchored, tetherin-fused Vpu could block the incorporation of tetherin(ΔGPI) into virions. Together these data indicate that in addition to surface downregulation/degradation, Vpu appears to have a further activity. Specifically, Vpu can reduce tetherin's antiviral function by inhibiting its localization to sites of viral assembly at the plasma membrane and its incorporation into virions.

ROLE OF UBIQUITINATION IN THE REMOVAL OF BST-2/TETHERIN FROM THE CELL SURFACE BY HIV-1 VPU.

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BST-2 restricts HIV-1 production by directly retaining nascent virions on the surface of infected cells. HIV-1 Vpu counteracts this restriction by decreasing the effective concentration of BST-2 at the cell surface. The optimal down-regulation of BST-2 depends on the interaction of Vpu with β-TrCP, the substrate adapter of a multi-subunit E3 ubiquitin ligase complex. This leads to the hypothesis that Vpu induces ubiquitination of BST-2 to induce trafficking events that remove BST-2 from the plasma membrane. Here, we observed that Vpu stimulates the ubiquitination of BST-2 in a manner dependent on its β-TrCP-binding sequence. Mutation of all potential ubiquitin acceptor sites in the cytoplasmic domain of BST-2 abrogated stimulation of ubiquitination by Vpu and was associated with a relative resistance to Vpu-mediated surface down-regulation. These data support a model in which Vpu induces the ubiquitination of BST-2, leading to the removal of BST-2 from its site of action as a virion-tethering factor and the relief of restriction.

INHIBITION OF HIV PARTICLE RELEASE BY BST-2 IS NEUTRALIZED BY A BST-2 SPECIFC ANTIBODY

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HIV-1 Vpu enhances virus release from the cell surface. This phenomenon is cell type dependent. Recent work identified BST-2/CD317/tetherin as a host factor whose inhibitory activity on viral release is counteracted by Vpu. A current model suggests that BST-2 inhibits virus release by physically tethering viral particles to the cell surface using its N-terminal TM domain and C-terminal gpi modification as anchors in the cell and viral membranes, respectively. Vpu is believed to counteract this inhibitory activity by down regulating BST-2 from cell surface. However, our own data suggest that in the context of an acute infection of T lymphocytes, BST-2 surface downregulation is not mandatory for efficient virus release. We have recently developed a high-titered antibody recognizing endogenous as well as exogenously expressed BST-2. The antibody was raised against the ectodomain of BST-2 and reacts well with cell-surface BST-2 in a variety of human cell types. We and others have found that structural features in the BST-2 ectodomain, incl. a coil-coil domain and three cysteines involved in protein-dimerization, are critical for BST-2 function. We hypothesized that antibody binding to BST-2 could affect the formation of cysteine-linked dimers and/or affect coil-coil-mediated protein-protein interactions and thus interfere with BST-2 function. Here, we analyzed the antagonistic potential of our BST-2antibody. Indeed, we found that treatment of transfected HeLa cells with BST-2 antibody effectively blocked the inhibitory effect of BST-2 on virus release. Interestingly, antibody-treatment not only increased the release of Vpu-deficient virus but enhanced the release of wt HIV-1 virions as well. This suggests that Vpu expressed from wt NL4-3 is not sufficient to fully negate the inhibitory effect of endogenous BST-2 in HeLa cells. BST-2 antibody-induced enhancement of virus release required pretreatment of the cells. Furthermore, kinetic data suggest that virus particles already tethered to the cell surface prior to antibody addition are not released. Therefore, we conclude that antibody neutralization of BST-2's tethering activity requires antibody binding prior to BST-2's engagement with budding viruses. Experiments will be presented to address the subcellular location where antibody interference occurs and to elucidate the precise mechanism of interference.

RAPID EVOLUTION OF NEF-MEDIATED TETHERIN ANTAGONISM IN A CHIMPANZEE EXPERIMENTALLY INFECTED WITH HIV-1

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It has been shown that switches between Vpu-, and Nef-mediated tetherin antagonism preceded the emergence of HIV-1 (Sauter et al., 2009). The precursors of HIV-1, SIVcpz from chimpanzees and SIVgor from gorillas, use Nef to counteract tetherin. Human tetherin, however, is resistant to Nef because of a deletion in its cytoplasmic region. This deletion most likely forced HIV-1 to switch from Nef to Vpu to counteract tetherin in the new human host. Notably, only pandemic HIV-1 M strains mastered this hurdle perfectly by switching from Nef- to Vpu-mediated tetherin antagonism. Currently, it is unknown how rapidly Nef or Vpu can acquire anti-tetherin activity in vivo and which domains in Nef are involved in tetherin antagonism. To address this, we analyzed nef alleles from a chimpanzee who had been experimentally infected with HIV-1 ten years earlier. In contrast to the nef genes of the original HIV-1 strains (SF2 and LAV) used for infection, the nef gene of the virus (JC16) obtained after ape passage antagonized chimpanzee (but not human) tetherin. Notably, the chimpanzee-adapted JC16 strain maintained the capability to counteract human and chimpanzee tetherin by Vpu. In the context of the infectious JC16 provirus, however, mainly Nef is used to antagonize chimpanzee tetherin. This is most likely because the proviral construct expresses Nef at higher levels than Vpu. The nef alleles derived from the chimpanzee differ in at least 25 amino acid residues from those of the input HIV-1 strains. Currently, we are mapping the changes underlying the gain of anti-tetherin function by HIV-1 Nef. Our data show that primate lentiviruses can regain lost anti-tetherin activities of their accessory genes within a single in vivo passage in a new host species. The analysis of these chimp-adapted HIV-1 nef alleles will give novel insights into the mechanism of Nef-mediated tetherin antagonism.

COMPARISON OF XMRV INFECTIONS IN HUMANS AND RHESUS MACAQUES

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XMRV is a human retrovirus first identified in prostate cancer tissues from men with a deficiency in the innate immunity gene, RNASEL/HPC1(Urisman et al., 2006). To gain insight into the consequences of XMRV infections, we performed studies that compared viral nucleic acid levels and cell tropism in men and in male and female adult rhesus macaques. FISH methods used SpectrumGreen labeled nucleic acid segments spanning the entire viral genome. IHC used a monoclonal antibody against SFFV Gag protein. In prostate cancer patients, XMRV nucleic acid and protein was present in the prostate only in stromal cells, mostly fibroblasts, but also in hematopoetic cells. Only about 1% of the stromal cells were positive for XMRV. Animal studies were performed with five healthy rhesus macaques (four males and one female) infected with XMRV intravenously. XMRV genomic RNA levels peaked at 7,500 copies per ml on day 7 pi in one animal. XMRV showed a wide dissemination of replicating virus even when the plasma viral load was undetectable. Isolated lymphoid cells and primarily CD4+ T cells were found positive in most lymphoid organs including spleen, lymph nodes and GI tract. The virus infection was, however, not restricted to bone marrow derived cells, but showed distinct target specificities in various organs. Foci of infected epithelial cells were detected in organs such as prostate, seminal vesicles and epididymis. In the lone female animal, XMRV positive epithelial and fibroblast like cells were detected in the vagina and cervix suggesting that the virus may be transmitted via sexual contact. Although XMRV dissemination was complete at day 6 pi, the prostate was positive by IHC only during the acute infection but contained during chronic infection in these healthy animals. Results show that XMRV has tropism for the prostate in both men and rhesus macaques. The animal studies show that in acute infection, XMRV localizes to the prostate epithelium while in chronically-infected humans the virus was present in the stroma suggesting the possibility of epithelial to mesenchymal transition. Our findings have implications for the possible role of XMRV in prostate cancer and other diseases.

THE HUMAN RETROVIRUS XMRV PRODUCES RARE TRANSFORMATION EVENTS IN CELL CULTURE BUT DOES NOT HAVE DIRECT TRANSFORMING ACTIVITY

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The xenotropic murine leukemia virus-related virus (XMRV) was first detected in human prostate cancer patients, and while it has been found to be associated with prostate cancer, no causality has been shown. We have investigated the ability of the XMRV envelope protein to transform 208F rat fibroblast cells, and found that although functional XMRV envelope could be produced in transfected 208F cells, no transforming activity was observed. We also investigated the ability of replicating XMRV to induce foci of transformation in cell culture. Virus was collected from HT-1080 cells infected with conditioned medium from 22Rv1 cells. The HT-1080 cells also contained an integrated copy of the LAPSN retroviral vector used as a marker of functional virus production. Only rare transformed foci were observed in XMRV-infected 208F rat fibroblasts, showing that the virus does not have acute transforming activity. Three foci of transformation were isolated, and all contained integrated copies of XMRV and produced virus. One of the three transformed 208F foci produced an acutely transforming virus generated by recombination between the LAPSN vector used in titration of virus and the mutant human NRAS genes from the HT-1080 cells used to produce XMRV. No transfer of human NRAS was observed in the other foci isolated, or from a fourth transformed 208F focus generated from virus made from HT-1080 cells without the LAPSN vector. This suggests that the transformed 208F cells that did not produce a transforming virus were transformed by insertional mutagenesis. Our results show that, as with other retroviruses, XMRV may have the ability to transform cells by activation of host cell oncogenes, but no direct transforming activity of the virus was observed.

COMPOUNDS THAT INHIBIT REPLICATION OF XMRV, A VIRUS IMPLICATED IN PROSTATE CANCER AND CHRONIC FATIGUE SYNDROME

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Xenotropic murine leukemia-related retrovirus (XMRV) is a recently discovered infectious agent [1] that has been linked to human prostate cancer [2] and chronic fatigue syndrome (CFS) [3]. Both diseases affect a large fraction of the world population, with prostate cancer affecting one in six men, and CFS affecting an estimated 0.4 to 1% of the population.

Forty-five compounds including twenty-eight drugs approved for use in humans were evaluated against XMRV replication in vitro. We will present data showing four of these compounds to be active against XMRV at sub-micromolar concentrations, in MCF-7 and LNCaP cells, a breast cancer and prostate cancer cell line, respectively. When combined these drugs displayed mostly synergistic effects against this virus, suggesting possible combination therapies to treat XMRV infections, thus delaying or preventing the selection of resistant viruses.

If XMRV proves to be a causal factor in prostate cancer or CFS, these discoveries may allow for rational design of clinical trials.

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SCREENING MOUSE GENOMES FOR XMRV-LIKE ELEMENTS

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XMRV represents the first reported case of a gammaretrovirus infection in humans. It bears very close resemblance to some of the endogenous xenotropic MLVs (Xmvs) found extensively in the genomes of inbred mice. XMRV was initially isolated from a subset of prostate cancer patients; it was then detected in a number of chronic fatigue syndrome patients as well as in some healthy subjects. Although its association with human disease is currently the subject of ongoing debate, the implication of cross-species transmission prompted us to study its possible origins.

Despite the high degree of sequence identity, the XMRV genome is clearly distinct from all Xmvs described so far. Different XMRV isolates from human tissues in several independent studies show very little variation in sequence and seem to tightly cluster together in phylogenetic analyses. Over the course of evolution Xmvs have successfully invaded the genomes of wild and inbred mice and a fraction of these elements have the ability to produce infectious virus. Given the high degree of similarity between XMRV and endogenous Xmvs, we hypothesized that there could be a reservoir of XMRV, or an ancestral virus that gave rise to XMRV, in mice. Although the provirus is not present in the sequenced mouse genome, there exists the possibility that other strains of mice might harbor XMRV-like sequences. We sought to determine whether we could find such elements in various mouse genomic DNA samples. We developed a highly sensitive PCR-based assay, using primers specific for regions of XMRV that show marked differences from some of the closest Xmvs described so far. We assessed the specificity of our assay by detecting XMRV-specific sequences in the genomic DNA from as few as 8 cells of a cell line chronically infected with XMRV, while not detecting any of the endogenous Xmvs that inhabit the C57BL/6 genome. Using this assay, we have screened genomic DNA samples from 70 different wild and inbred mouse strains that represent a total of 18 *Mus* species. None of the tested samples yielded an XMRV-specific sequence, strengthening the argument against the possibility of a laboratory contaminant giving rise to XMRV.

We are also interested in characterizing an endogenous Xmv that is a close relative of XMRV, namely Xmv-43 (Bxv-1). Currently, studies are underway to compare infectivity and replication properties of Bxv-1 with those of XMRV.

DEVELOPMENT OF A MULTIPLEX SEROLOGICAL ASSAY TO DETECT XMRV ANTIBODIES

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Recent studies of the role of XMRV in prostate cancer or chronic fatigue syndrome (CFS) have provided conflicting evidence for the association of the virus with these conditions. There are also limited and variable reports of the prevalence of the virus in healthy donor populations. As an initial step in characterizing the sero-prevalence of XMRV in both disease states and in healthy populations, we initiated the development and validation of a multiplex, ELISA-based test.

Each XMRV viral gene was individually expressed in different systems and purified under different conditions to simultaneously evaluate the optimal antigen(s) for this platform. Using pooled human normal plasma (negative for HIV-1/2, HCV, HTLV-I/II and HBV), normal un-screened donor plasma and sera, and clinical specimens from patients XMRV-positive (by a qPCR, nested PCR or antibody test), assay conditions were rigorously defined and antigens with antibody reactivity were down-selected for further validation and inclusion in the Meso Scale Discovery multiplex format. The test includes a negative control antigen to reduce the possibility of false positives. Using this platform, initial studies have detected XMRV antibodies in a majority of samples from individuals previously shown to be positive by PCR. Final test validation is on-going and results from a screen of >1,000 samples will be discussed.

INVESTIGATIONS INTO XENOTROPIC MURINE LEUKAEMIA VIRUS-RELATED VIRUS INFECTION

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Xenotropic murine leukaemia virus-related virus (XMRV) is a newly discovered gammaretrovirus that was first identified from prostate cancer samples and has recently been reported to be present in 67 % of patients with chronic fatigue syndrome (CFS). We are interested in defining the host cell tropism of this virus as well as studying the link to human disease.

As XMRV was recovered from PBMCs, which express several known anti-viral restriction factors, this raised the possibility that like HIV, XMRV may have acquired resistance to restriction. We therefore investigated the susceptibility of XMRV to a panel of different restriction factors. We found that both human APOBEC3 and tetherin proteins, but not human TRIM5α, were able to block XMRV replication. In addition, the virus was inhibited by factors from non-human species including mouse Apobec3, tetherin and Fv1 proteins. These results have important implications for predicting the natural target cells for XMRV replication, for relating infection to viral pathogenicity and pathology, as well as for the design of model systems with which to study XMRV-related diseases.

In addition to this work we have examined 170 UK CFS patient samples and 385 controls for evidence of XMRV infection. We have not identified XMRV DNA in any samples by quantitative PCR (0/321) and although some serum samples showed XMRV neutralising activity (26/565) only one of these positive sera came from a CFS patient. Therefore, we have not observed any association between XMRV infection and CFS. Most of the neutralising sera showed significant cross-reactivity in serological responses. However, four positive samples were specific for XMRV in our assays, indicating that XMRV infection may occur at low levels in the general population, although with currently uncertain outcomes.

A LAYERED STRUCTURE IN THE HIV-1 GP120 INNER DOMAIN THAT REGULATES GP41 INTERACTION AND THE TRANSITION INTO THE CD4-BOUND CONFORMATION IS CONSERVED AMONG SIV ENVELOPE GLYCOPROTEINS

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CD4 binding triggers conformational changes in the human immunodeficiency virus (HIV-1) gp120 envelope glycoprotein that promote its interaction with one of its chemokine receptors, CCR5 or CXCR4. CD4 binding ultimately leads to viral-cell membrane fusion mediated by the gp41 transmembrane envelope glycoprotein. We have recently reported that a key network of interactions that modulate the interaction of layers in the inner domain is important for gp120-gp41 association and regulates CD4 as well as CCR5 and 17b binding (1). Importantly, based on sequence alignment, this network is conserved among the envelope glycoproteins of primate lentiviruses. We have now extended our analysis to the simian immunodeficiency virus (SIV) gp120. In SIV this network also regulates gp41 interaction but in a slightly different manner than in HIV-1 gp120. These differences may compensate for the presence of a tryptophan residue in the Phe 43 cavity of SIV envelope glycoproteins; this modification has been shown to be sufficient to induce the adoption of a CD4-bound conformation. Consistent with the necessity to balance the spontaneous adoption of such conformation by SIV gp120, we observed that the inner domain layers of SIV modulated CD4 binding in a manner different than that in HIV-1. Thus, a key network of interactions in the inner domain of primate lentivirus envelope glycoproteins modulates gp41 interaction and negotiates conformational transitions from and towards the CD4-bound state.

(1) Finzi et al., Molecular Cell (2010), doi:10.1016/j.molcel.2010.02.012

STRUCTURAL BASES OF RETROVIRAL INTASOME ASSEMBLY, ACTIVITY AND INHIBITION

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A retrovirus must integrate a DNA replica of its genome into a host cell chromosome as an obligatory step of its lifecycle. This process is orchestrated by integrase (IN), an enzyme that binds both termini of a viral DNA molecule and catalyzes two distinct reactions. Firstly, in 3′ processing, a di- or tri-nucleotide is removed from the 3′ ends of viral DNA and secondly, in strand transfer, the two newly processed ends are inserted in a concerted fashion into the genomic DNA of the host.

We recently reported a 2.9 Å crystal structure of full-length IN from the prototype foamy virus (PFV) in complex with pre-processed viral DNA mimics. The structure reveals the organization of the retroviral intasome comprising an IN tetramer tightly associated with a pair of viral DNA ends. All three canonical IN structural domains are involved in extensive protein-DNA and protein-protein interactions. The viral DNA termini are partly melted, and the 3′-OH group of the invariant CA dinucleotide is involved in coordination of a metal cofactor.

Herein we further report the structure of this enzyme bound to unprocessed viral DNA and two metal cofactors, thereby revealing the structural basis for the first step of retroviral integration.

Soaking clinical strand transfer inhibitors Raltegravir and Elvitegravir into the original crystals revealed their mechanism of action. The conserved metal chelating and halobenzyl groups of these compounds cooperate to displace the reactive 3′ viral DNA end from the active site, disarming the viral nucleoprotein complex. We will present additional structures and discuss the mechanism of drug resistance via the N155H escape pathway.

LENTIVIRAL VECTOR MEDIATED GENE THERAPY FOR BETA-THALASSEMIA: TRANSFUSION INDEPENDENCE AND ACTIVATION OF HMGA2

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We report a successful use of lentiviral vector gene therapy in the treament of beta-thalassemia, which was accompanied by a clonal expansion involving integration of the lentiviral vector in the HMGA2 gene. The hemoglobinopathies are the most prevalent genetic diseases worldwide. An 18 year old male with severe $ß^{E}/ß^{0}$-thalassemia dependent on monthly transfusions was treated *ex vivo* with a lentiviral vector expressing a marked $ß^{A\text{-}T87Q}$-globin gene. Twenty two months post-transplantation, hemoglobin (Hb) levels are 9.5 g/dL, of which ~ 3 g/dL contains $ß^{A\text{-}T87Q}$-globin, the remainder being HbE and HbF. No transfusion has been provided for more than one year, and there are near physiological levels of $ß^{A\text{-}T87Q}$-globin expression on a per gene basis. Much of the therapeutic effect derives from a dominant cell clone with vector insertion within the HMGA2 gene. The vector is integrated in the third intron of the gene resulting in formation of a chimeric message containing 5' HMGA2 sequences and a new 3' end derived from the lentiviral vector. As a consequence, negative acting microRNA binding sites in the 3' end of the normal message were not present in the chimeric form. Analysis of message levels indicated that the chimeric message is overexpressed, probably due to a combination of release from microRNA control and also an increased rate of transcription initiation. Expression of HMGA2 is associated with increased stem cell persistance and HMGA2 rearrangments that remove microRNA binding sites are associated with benign tumors. The subject to date remains transfusion independent and thus continues to benefit from gene therapy. This study provides an example of clonal expansion in a lentiviral gene therapy trial, where the vector may have contributed to cell growth or persistence.

DIVERSE FAMILIES OF NON-RETROVIRAL VIRUSES IDENTIFIED AS ENDOGENOUS ELEMENTS IN AVIAN AND MAMMALIAN GENOMES.

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Integration of viral genes into germline cells can lead to vertical inheritance of viral genetic material. Integration is primarily a characteristic of retroviruses, but has also been described in other virus groups. We carried out systematic screening of mammalian and avian genomes to identify sequences derived from non-retroviral viruses. Screening led to the detection of endogenous sequences derived from five viral families, including single stranded DNA viruses, negative sense RNA viruses and DNA reverse transcribing viruses. Endogenous virus-like elements (EVLEs) occurred as complete genomes, single genes, and isolated gene fragments. Comparative sequence analysis indicated that EVLEs derived from RNA-viruses were generated through interactions with cellular retroelements, whereas virally mediated integration was implicated for those derived from DNA viruses. Characterization of EVLE distribution and diversity allowed the minimum ages of the represented virus groups to be estimated, and indicated likely reservoir host species for their contemporary exogenous members. Phylogenetic analysis revealed that some EVLEs represent novel virus groups that may have contemporary counterparts. This study provides the first evidence that viral genetic material derived from both DNA and RNA viruses can stably enter the host germline, a discovery that greatly broadens the scope of paleovirological investigations.

CAPSID-SPECIFIC RETROVIRAL RESTRICTION FACTOR TRIM5 IS A MULTIFUNCTIONAL E3 UBIQUITIN LIGASE THAT PROMOTES INNATE IMMUNE SIGNALING

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Immediately following retroviral invasion of the target cell cytoplasm, the capsid of retroviruses is engaged by TRIM5, a germ-line encoded host restriction factor. Recognition of the hexagonal capsid lattice requires either of two domains – PRYSPRY or cyclophilin - depending upon the TRIM5 orthologue. Antiviral potency correlates with avidity for the capsid but the mechanism of restriction is unknown. Conditional requirement for its RING finger E3 ubiquitin ligase domain and B-Box-type zinc finger protein-protein interaction domain, host cell-dependent activity, suppression by drugs that block signal transduction, and induction by interferon suggest that TRIM5 activity requires cellular cofactors and signal transduction, properties reminiscent of innate immune receptors for pathogen associated molecular patterns (PAMPs). We found that TRIM5 disruption in THP1 macrophages or monocyte-derived dendritic cells rescued HIV-1, SIV, Vesicular Stomatitis Virus, or New Castle Disease Virus from the antiviral state established by TLR agonists. TRIM5 stimulated NF-kB and AP1/Jun transcription, and cooperated with IRF3 to activate IFNbeta. These activities required the RING finger and B-Box and were observed with all TRIM5 orthologues, but not close paralogues. TRIM5 associated with TAK1 and TRAF2, stimulated the phosphorylation of TAK1, IKKs, and JNK, and contributed to transcriptional induction of inflammatory chemokines, cytokines and interferon-stimulated genes. In addition to its ability to function as an E3 ubiquitin ligase forming ubiquitin-K48 linkages, TRIM5 catalyzed the assembly of free ubiquitin chains linked via K63, and the free ubiquitin chains contributed to TAK1 activation. The effects of TRIM5 overexpression or knockdown were mimicked by similar perturbations of TAK1 or the K63-specific E2 ubiquitin ligase Ubc13. Challenge of THP1 cells, monocyte-derived macrophages, or dendritic cells with retroviruses induced transcription of inflammatory genes, the magnitude of which correlated with the avidity of the particular TRIM5-capsid combination. These findings demonstrate that TRIM5 stimulates innate immune signaling by catalyzing the assembly of free K63-ubiquitin chains that activate TAK1, and that TRIM5 has the properties of a pathogen associated molecular pattern receptor specific for the hexagonal retroviral capsid lattice.

A CRYPTIC SENSOR FOR HIV-1 ACTIVATES INNATE IMMUNITY IN DENDRITIC CELLS

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Dendritic cells (DC) serve a key function in host defense, linking innate detection of microbes to the activation of pathogen-specific adaptive immune responses. Whether there is recognition of a HIV-1 Pathogen-Associated Molecular Pattern (PAMP) by host innate Pattern-Recognition Receptors (PRR) and subsequent coupling to antiviral T cell responses is not yet known. DC are largely resistant to infection with HIV-1, but this host restriction can be overcome by co-infection with SIVmac pseudovirions containing the Vpx protein.

We found that, in such circumstances, HIV-1 induces a type-I interferon response. By using anti-retroviral drugs and viral mapping, we found that this innate response occurs after integration and requires Gag synthesis. We further found that Capsid (CA) mutants T54A/N57A and Q63A/Q67A had an increased ability to induce an innate response, while the cylcophilin A (CypA)-binding mutant G89V was defective in this process. Cyclosporin A (CsA) treatment, delayed after viral exposure to maintain infectivity, inhibited the induction of the innate response. Inhibition of CypA expression using shRNA also prevented the induction of the response. This indicates that the innate response is dependent on the interaction of newly-synthesized HIV-1 CA with cellular cyclophilin A (CypA). Because the peptidyl-prolyl isomerase CypA also interacts with CA to promote HIV-1 infectivity, our results suggest that CA conformation has evolved under opposing selective pressures for infectivity versus furtiveness.

The transcription factor IRF3 induces type-I interferon expression following activation of PRRs such as TLR3 and RIG-I. By using shRNA, we demonstrated that the innate response to HIV-1 also requires IRF-3. By using T cell clones and HLA-matched DC, we further found that infection of dendritic cells with HIV-1 in the presence of Vpx, and induction of an innate immune response, leads to potent activation of HIV-specific T cells.

HIV-2 is less pathogenic than HIV-1 and this is associated with control of the infection by the host immune system. Unlike HIV-1, HIV-2 expresses Vpx. We found that HIV-2 is able to infect DC and also to induce an innate immune response. Thus, an innate response to HIV-1 exists, but is cryptic in the absence of DC infection. The lack of induction of this response in HIV-1-infected individuals may contribute to the failure the immune system at controlling HIV-1 infection. Manipulating this response may be essential to generate a HIV-1 vaccine.

VPU-MEDIATED ENHANCEMENT OF HIV-1 RELEASE IS SEPARABLE FROM VPU-MEDIATED DEGRADATION OF HOST RESTRICTION FACTOR CD317 (TETHERIN)

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The HIV-1 accessory protein Vpu, an 81-amino-acid integral membrane protein, enhances virus particle release by counteracting host restriction factor(s) that retain budded virions on the surface of infected cells. Recently, BST-2/CD317 (also named tetherin), was identified as the host restriction factor whose depletion enhances Vpu-defective virus release. Human tetherin blocks the release of not only HIV-1 but also that of a variety of other enveloped viruses. Vpu enhances virus release by counteracting tetherin. Recent studies have demonstrated that tetherin variants from mice, rhesus macaques (rh), and African green monkeys (agm) were able to inhibit HIV-1 particle release, but were resistant to antagonism by Vpu. In the present study, we investigated the mechanism of Vpu-mediated antagonism and its specificity for tetherins from human, mouse, rh, and agm. We observed that the antagonism of tetherin by Vpu is associated with proteasome-mediated degradation, as MG132 and ALLN inhibit Vpu-mediated tetherin degradation. Lysine-mediated ubiquitylation of tetherin is not required for tetherin degradation, as multiple-lysine mutants also undergo degradation by Vpu. The resistance of non-human tetherin proteins to antagonism by Vpu correlates with the absence of Vpu-mediated degradation. Recent studies show that in agm kidney cells (COS) human tetherin is poorly expressed compared to 293T cells. Surprisingly, we observed that Vpu markedly increases levels of human tetherin expression in COS cells, suggesting a Vpu-mediated stabilization of human tetherin in this cell type. This stabilization of tetherin by Vpu is specific for human tetherin, and maps to the transmembrane sequence, as chimeric tetherins containing rh and agm transmembrane sequences are not stabilized by Vpu. Despite the Vpu-mediated stabilization of human tetherin levels in COS cells, Vpu is still able to enhance HIV-1 release from this cell type. These observations demonstrate that Vpu-mediated enhancement of HIV-1 release and Vpu-mediated tetherin degradation are two separable phenomena. COS cells thus provide a system for the molecular dissection of Vpu and tetherin function.

VPU-MEDIATED DEGRADATION OF CD317 AND CD4, BUT NOT HIV-1 RELEASE ENHANCEMENT, DEPEND ON B-TRCP2

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HIV-1 counteracts the virion release restriction imposed by CD317 by expressing Vpu, yet the mechanism of this antagonism is not understood. Vpu targets the CD4 receptor for proteasomal degradation by bridging it to β-TrCP, a component of an E3 ubiquitin ligase complex. Recently, it has been suggested that β-TrCP is also critically involved in Vpu's ability to overcome the CD317-mediated virion release block.
To test this model we analyzed the consequences of several experimental strategies to interfere with the Vpu-β-TrCP protein-protein interaction. Under these conditions, we, in parallel, studied effects of Vpu on expression and localization of CD317 and CD4, as well on its ability to promote HIV-1 release. Our results demonstrate a strict requirement for Vpu's di-serine motif S52/S56 for degradation of CD4 and CD317, reduction of cell surface exposure of CD317, and HIV-1 release enhancement. They further show a critical role of β-TrCP2, but not of the structurally related β-TrCP1, for Vpu-mediated degradation of both receptors. In β-TrCP2-depleted cells, however, Vpu remained fully active to downregulate CD317 from the cell surface and to overcome the HIV-1 release restriction. Furthermore, Vpu remained capable to efficiently antagonize degradation-insensitive CD317 variants and this correlated with a preserved ability to downregulate the restriction factor from the cell surface.
We conclude that Vpu can overcome the release restriction in the absence of a β-TrCP-dependent depletion of intracellular pools of CD317, indicating cell surface downmodulation of the restriction factor as the key mechanism of Vpu antagonism. Thus, β-TrCP is dispensable for the Vpu antagonism to CD317-mediated restriction of HIV-1 particle release. Together, these findings predict that the critical di-serine motif of Vpu in its phosphoryplated state, besides interacting with β-TrCP, also binds to yet unidentified host cell factor(s) with essential roles in HIV-1 release enhancement.

HOST ADAPTATION OF HIV-1 VPU

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The HIV-1 Vpu protein enhances the release of viral particles from the cell surface in a cell-type specific manner. In the absence of Vpu, nascent virions remain tethered to the cell surface in restricted cell types. Recently, a human host factor, BST-2/CD317/tetherin was found to be responsible for the inhibition of virus release. It was also reported that HIV-1 Vpu can target human BST-2 but is unable to interfere with the function of murine or monkey BST-2. Previous work demonstrated that the TM domains of both Vpu and BST-2 are critical for antagonism of BST-2. The TM domain of the Vpu-sensitive human BST-2 carries a two amino acid insertion and 7 substitutions relative to the Vpu-insensitive rhesus BST-2. We performed a gain-of-function study to determine, which of these differences in the BST-2 TM domain account for the differential sensitivity to Vpu. We transferred human BST-2 TM sequences into the rhesus BST-2 TM domain and assessed the resulting chimeras for inhibition of HIV-1 virus release and sensitivity to Vpu. We found that a single-amino-acid change in the rhesus BST-2 TM domain was sufficient to confer Vpu sensitivity. In an extension of that study we analyzed Vpu sequences from serially passaged SHIV isolates. SHIV viruses are chimeric viruses carrying Vpu and Env sequences from HIV-1 in the backbone of SIVmac239. Virus from an animal exhibiting increased pathogenicity contained mutations in Vpu relative to the input SHIV. Replication studies in rhesus PBMC of this virus and a vpu-defective variant revealed profiles similar to those of Vpu+/- HIV-1 in human cells suggesting the SHIV Vpu protein had acquired the ability to target rhesus BST-2. Experiments in 293T cells expressing human and rhesus BST-2, respectively, confirmed that the SHIV Vpu isolate had gained the ability to target rhesus BST-2. This gain of function was accompanied by a partial loss of activity towards human BST-2 and mapped to the Vpu TM domain. In conclusion, we identified two novel gain-of-function mutants. One is a BST-2 mutant which can be antagonized by NL4-3 Vpu and the other is a Vpu mutant that can enhance the release of NL4-3 particles in the presence of rhesus BST-2. These results highlight the critical importance of the TM domains for Vpu-BST-2 functional interaction and demonstrate that Vpu's ability to enhance virus release is under positive selection pressure *in vivo*.

IDENTIFICATION OF A DOMAIN IN THE MEMBRANE-PROXIMAL HELIX OF THE CYTOPLASMIC TAIL OF VPU REQUIRED TO OVERCOME TETHERIN-MEDIATED RESTRICTION OF HIV-1 PARTICLE RELEASE

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The human immunodeficiency virus type 1 (HIV-1) Vpu protein promotes CD4 downregulation and enhances particle release from restrictive cell types. The effect of Vpu on particle release is mediated through its effects on tetherin, an interferon-inducible host restriction factor that potently retains particles on the plasma membrane. The mechanism by which Vpu elicits relief of tetherin-mediated restriction to particle release is not yet clear. The cytoplasmic tail of Vpu contains signals responsible for intracellular targeting and colocalization of Vpu with tetherin in the trans-Golgi network (TGN). We undertook a scanning mutagenesis approach to identify residues in the Vpu cytoplasmic tail that are critical to relief of tetherin-mediated restriction of particle release. Substitution of residues 42-46 within helix two of Vpu (the membrane-proximal helix of the cytoplasmic tail) abrogated particle release. Three site-specific substitutions within this region were identified (D43R, R44E, and L45S) that resulted in substantial impairment in the ability of Vpu to overcome tetherin's effects. Surprisingly, all three of these mutants retained their ability to downregulate cell surface tetherin. In contrast, the S52A/S56A double mutant was impaired in downregulation of cell surface tetherin, but retained the ability to enhance particle output of Vpu-deficient virus. This study identifies a key region in the Vpu cytoplasmic tail involved in overcoming tetherin's effects, and points out a discordance between the ability of Vpu to overcome tetherin-mediated restriction and its effects on downregulation of cell surface levels of tetherin.

ANALYSIS OF INDIVIDUAL AMINO ACIDS WITHIN THE BST-2 AND HIV-1 VPU TRANSMEMBRANE DOMAINS IN THE CONTEXT OF SIMIAN-HUMAN IMMUNODEFICIENCY VIRUSES (SHIV).

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Pathogenic simian-human immunodeficiency viruses (SHIV) contain HIV-1 Vpu and SIV Nef, both shown to counteract BST-2 inhibition of virus release in a species-specific manner. Analysis of BST-2 sequences isolated from multiple rhesus and pig-tailed macaques displayed an increase in sequence variability among rhesus versus pig-tailed macaques. We show that human and pig-tailed BST-2 proteins restrict SHIV release and are susceptible to the Vpu and Nef proteins. We found that sequential "humanization" of the pig-tailed BST-2 (ptBST-2) protein resulted in a fluctuation in the sensitivity to Vpu. Mutations with the most significant impacts during sequential "humanization" were: i) substitution of the valine at position 38 with an isoleucine; and ii) mutation of the leucine at position 41 to an isoleucine. We also tested the hypothesis that the length of the transmembrane domain was a determinant of susceptibility using a human BST-2 with the leucine and glycine residues at positions 24 and 25 either deleted or substituted with alanines or isoleucines and a ptBST-2 with an insertion of a leucine and glycine at positions 29 and 30. Our results show that the length of the transmembrane (TM) domain in human and ptBST-2 proteins is important for BST-2-mediated restriction and susceptibility to viral proteins rather than the hydrophobicity of the amino acids present. We show that substitution of the invariant tryptophan in the HIV-1 Vpu TM domain with an alanine abrogated the enhanced virion release function, but only slightly diminished Vpu-mediated CD4 down-regulation. Mutation of residues surrounding the tryptophan residue also decreased the ability to enhance virion release, but had no impact on the CD4 down-regulation. Finally, we show that exchange of the HIV-1 Vpu TM domain with that of the M2 protein of influenza A virus resulted in a diminished capacity to enhance virion release in Hela cells. Taken together, our results show that SHIV can be used as a model to study both human and non-human primate BST-2 mechanisms of restriction and emphasize the importance of the tertiary structure in this restriction. We provide evidence for possible alternate mechanisms of HIV-1 Vpu in enhancing virion release in vivo separate from BST-2 antagonism.

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COMPENSATORY CHANGES IN GP41 CONFER RESISTANCE TO TETHERIN IN A PATHOGENIC DERIVATIVE OF A NEF-DELETED SIV VACCINE STRAIN

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Nef is the viral gene product in SIV that counteracts restriction by tetherin (BST2, CD317 or HM1.24), an interferon-inducible host-cell factor that inhibits the detachment of virus particles from infected cells. A role for Nef in opposing restriction by tetherin may help to explain the attenuated phenotype of *nef*-deleted SIV. However, since Nef is a polyfunctional protein, the loss of other functional activities may also contribute to attenuation. Macaques infected with *nef*-deleted strains of SIV typically control virus replication and do not develop AIDS. However, under certain circumstances, *nef*-deleted SIV may regain the ability to replicate to moderate levels and cause disease.

We found that a pathogenic derivative of a *nef*-deleted SIV vaccine strain regained resistance to rhesus tetherin. Similar to wild-type SIV, the ability of this virus to antagonize tetherin was species-specific, since it remained susceptible to human tetherin. Resistance to rhesus tetherin mapped to amino acid changes in the cytoplasmic tail of gp41 and, similar to HIV-2 Env, depended on the presence of a conserved tyrosine-based endocytosis motif in the cytoplasmic tail of gp41. The compensatory changes also resulted in the co-localization of SIV Env with tetherin in infected primary rhesus macaque CD4+ T cells.

These observations suggest that the ability to antagonize tetherin is important for lentiviral pathogenesis, since this activity was restored in a *nef*-deleted strain of SIV that regained a pathogenic phenotype *in vivo*.

SEQUENCES IN GALV ENV THAT CONFER SENSITIVITY TO VPU

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HIV-1 efficiently forms pseudotyped particles with Murine leukemia virus (MLV) Env but not by the related Gibbon ape leukemia virus Env (GaLV) or a chimeric MLV Env with the GaLV cytoplasmic tail. We have determined that this is incompatibility is modulated by the HIV-1 accessory protein Vpu. In this study, we sought to determine the modular sequence in the GaLV Env cytoplasmic tail required for this restriction in the presence of Vpu. With a series of truncation and point mutants in the chimeric Env, we have identified that the sequence conferring sensitivity to Vpu is 16 amino acids in length extending from the -25 to the -9 positions of the C terminal cytoplasmic tail. This region is predicted to form an alpha helix. Disruption of this proposed helix by the addition of Proline at any of multiple positions results in a protein that remains infectious but is no longer sensitive to Vpu. The restriction by Vpu is phosphorylation independent as the conserved tyrosine can be mutated without losing Vpu sensitivity. Using an Alanine scan we have determined the motif that makes GaLV Env Vpu sensitive is INxxIxxVKxxVxRxK. While many of these positions can be replaced with amino acids with similar biophysical properties without disrupting the Vpu sensitivity, the final lysine residue is absolutely required. This Vpu sensitivity sequence appears to be modular as the unrelated Rous Sarcoma Virus (RSV) Env with a GaLV cytoplasmic tail is also Vpu sensitive. In addition, MLV Env can be made Vpu sensitive by mutating 2 amino acids in its cytoplasmic tail to make it more closely resemble the Vpu sensitivity motif. This Vpu target sequence is contained in other host cellular transmembrane proteins and may be targeted by Vpu. These data will help elucidate mechanisms of host protein modulation by Vpu and possibly identify novel cellular Vpu targets.

REQUIREMENT OF THE HUMAN T-CELL LEUKEMIA VIRUS P12 AND P30 GENES FOR INFECTIVITY OF HUMAN DENDRITIC CELLS AND MACAQUES BUT NOT RABBITS

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The identification of the viral genes necessary for HTLV-1 persistence in humans may provide targets for novel therapeutic approaches. We ablated the p12, p30, or HBZ genes in HTLV-1 and tested the infectivity of the mutant viruses in human dendritic cells, rabbits, and macaques. All these viral mutants retained their ability to infect rabbits, the absence of HBZ reduces virus levels and no reversion of the point mutations introduced in the genome of the viral mutants was observed. In contrast, none of the four macaques inoculated with p12 knock out virus were infected; some of the macaques inoculated with the HBZ knock out or the p30 knock viruses selected within weeks from infection wild type virus and had serum antibodies to HTLV-1 proteins. The impairment of infectivity in macaques paralleled the infectivity of these mutant viruses in human dendritic cells in vitro, as neither the p30 nor the p12 knock out viruses were able to productively infect human dendritic cells. Collectively, these data demonstrate that the p30 and p12 genes are essential for infectivity of dendritic cells and suggest that infection of this cell type is important for the initiation and maintenance of HTLV-1 infection in primate species.

CCR5 KNOCKOUT IN HUMAN HSC AS AN ANTI-HIV THERAPY AND STRAIN-SPECIFIC EFFECTS OF R5-TROPIC HIV-1 INFECTION.

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CCR5 is the major cellular co-receptor used by HIV-1 and an important target for both drug and gene based anti-HIV therapies. Using CCR5 targeted zinc finger nucleases (ZFN) we can permanently disrupt the CCR5 gene in human hematopoietic stem cells (HSC) at high efficiency. Transplantation of ZFN-treated HSC into NSG mice leads to the generation of CCR5-negative HIV-resistant human hematopoietic progeny that completely suppress CCR5-tropic HIV-1 replication *in vivo*. Such findings suggest that ZFN treatment of autologous HSC could be used to control virus replication in HIV patients, and our progress towards this clinical application will be presented.

Using ZFNs, we are also able to achieve site-specific gene addition at a disrupted CCR5 locus through homologous recombination (HR) repair. This allows the possibility of inserting additional anti-HIV genes at the CCR5 locus, thereby protecting cells that are only modified at a single CCR5 allele, as well as providing protection against CXCR4-using viruses. Such site-specific gene addition should also result in more predictable expression of therapeutic genes and reduce the potential for insertional mutagenesis.

Humanized NSG mice are a powerful new model system in which to evaluate anti-HIV therapies and study HIV-1 pathogenesis. As part of our studies of this anti-CCR5 gene/cell therapy, we noted differential effects of a panel of R5-tropic viruses on the rate and extent of human T cell depletion in various tissues, including the thymus and GALT, and on overall levels of immune activation in the human cells. Interestingly, we observed a correlation between the pathogenicity of an R5-tropic virus and its ability to up-regulate CCR5 expression in the thymus, peripheral blood, spleen and GALT. We propose that such CCR5 up-regulation results in the depletion of cells from tissues that do not normally have a large population of CCR5-expressing lymphocytes, and increases the pathogenic consequences of R5-tropic infections.

POTENT DOWN-REGULATION OF CCR5 BY RNA INTERFERENCE PROTECTS CD4+ T CELLS FROM HIV INFECTION IN THE HU-BLT MOUSE MODEL.

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Inhibiting the expression of the HIV-1 co-receptor CCR5 holds great promise for controlling HIV-1 infection in patients. Here we report stable knockdown of human-CCR5 by a short hairpin RNA (shRNA) in a humanized bone marrow/liver/thymus (BLT) mouse model. We delivered a potent shRNA against CCR5 into human fetal liver-derived CD34+ hematopoietic progenitor/stem cells (HPSCs) by lentiviral vector transduction. We transplanted vector-transduced HPSCs solidified with matrigel and a thymus segment under the mouse kidney capsule. Vector-transduced autologous CD34+ cells were subsequently injected in the irradiated mouse intended to create systemic reconstitution. CCR5 expression was down-regulated in human T cells and monocytes/macrophages in systemic lymphoid tissues, including gut associated lymphoid tissue (GALT), the major site of HIV-1 replication. The shRNA-mediated CCR5 knockdown had no apparent adverse effects on T cell development as assessed by polyclonal T cell receptor Vβfamily development and naive/memory T cell differentiation. CCR5 knockdown in the secondary transplanted mice suggested the potential of long-term hematopoietic reconstitution by the shRNA transduced HPSCs. We next examined HIV inhibition ex vivo and in vivo. CCR5 tropic HIV-1 infection was effectively inhibited in isolated human splenocytes ex vivo. When mice were challenged with CCR5 tropic HIV-1, we observed selective maintenance of CCR5-shRNA expressing CD4+ T cells in various tissues including GALT in vivo. These results demonstrate that lentiviral vector delivery of shRNA into human HPSCs could stably down-regulate CCR5 and prevent CD4+ T cell depletion in vivo. Our current results provide further evidence that it may be possible to create a single administration reagents, using a lentiviral vector expressing CCR5 shRNA through hematopoietic stem cell transduction and transplantation, to stably control HIV-1 infection.

EFFICIENT NEF-MEDIATED DOWNMODULATION OF TCR-CD3 IS ASSOCIATED WITH HIGH CD4+ T CELL COUNTS IN VIREMIC HIV-2-INFECTED INDIVIDUALS

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It is currently under debate whether Nef-mediated down-modulation of TCR-CD3 protects SIV- and/or HIV-2-infected hosts against damaging high levels of immune activation and the loss of CD4+ T cells. To further address this question, we cloned nef alleles from 20 viremic (plasma viral load >500 copies/ml) and 16 non-viremic (<500 copies/ml) HIV-2-infected individuals into proviral HIV-1 IRES/eGFP constructs and analyzed their effect on CD4, CD3, CD28 and class I MHC cell surface expression, activation and apoptosis of primary CD4+ T cells. We found that the potency of nef alleles derived from viremic individuals in down-modulating TCR-CD3 correlated significantly with high numbers of CD4+ T cells in vivo and with low levels of activation and apoptosis of virally infected primary CD4+ T cells in vitro. No such correlations were observed in non-viremic HIV-2-infected individuals and for other Nef functions. None of these nef alleles antagonized human tetherin, although several showed significant activity against the tetherin variant found in the original host of HIV-2, the sooty mangabey. Altogether, our data suggest that many HIV-2-infected individuals do not progress to AIDS irrespectively of Nef function because they efficiently control the virus. However, the ability of Nef to remove TCR-CD3 from the cell surface may help HIV-2-infected individuals with relatively high viral loads to maintain normal CD4+ T cell counts by preventing activation-induced cell death, just as previously observed in SIVsmm-infected sooty mangabeys.

LA PROTEIN IS INVOLVED IN GLYCOSYLATED GAG (GPR80^{GAG})-FACILITATED RELEASE OF MOLONEY MURINE LEUKEMIA VIRUS (M-MULV)

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Murine leukemia viruses (MuLVs) and other gammaretroviruses encode a unique form of Gag polyprotein, gPr80^{gag} or glyco-gag, by initiation of translation from an upstream initiation site in the same reading frame as the Pr65^{gag} Gag precursor. gPr80^{gag} is evolutionarily conserved among gammaretroviruses, and there is strong selection for recovery of gPr80^{gag} if mutant virus is infected into animals. We previously reported that gPr80^{gag} facilitates release of virions from cells along an IFN-sensitive pathway (Nitta et al., PNAS 2010). In particular, gPr80^{gag}-facilitated release occurs through lipid rafts, because gPr80^{gag}-negative M-MuLV has lower cholesterol content (higher buoyant density), it is less sensitive to inhibition of release by the cholesterol-depleting agent MßCD, and there is less Pr65^{gag} associated with detergent-resistant membranes (DRMs) in mutant-infected cells. In recent studies, we found that a plasmid expressing only the N-terminal 88 amino acids of gPr80^{gag} (pMuLV-gg88) shows full activity in facilitating release of an MuLV vector (AKAQ188) from transiently transfected 293T cells. To further understand the mechanism of gPr80^{gag} action we sought to identify cellular proteins that might be involved. We performed a yeast two-hybrid screen with a bait plasmid consisting of the N-terminal unique region of gPr80^{gag} fused to bacterial LexA protein on a human cDNA library. Only one cDNA was identified corresponding to the cellular protein La/SSB – a protein associated with several aspects of RNA metabolism such binding of Pol III transcripts. In vivo interaction between gPr80^{gag} and La was evident from relocalization of human or mouse La into the cytoplasm of cells transfected with pMuLV-gg88. Overexpression of mouse or human La could substitute for pMuLV-gg88 in enhancing release of MuLV virions from 293T cells transiently transfected with AKAQ188. The resulting virus showed a buoyant density characteristic of higher cholesterol content, and DRMs from La-overexpressing cells showed higher Pr65^{gag} content. These results are consistent with gPr80^{gag} functioning by recruiting La to the cytoplasm.

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VISITOR INFORMATION

EMERGENCY	CSHL	BANBURY
Fire	**(9) 742-3300**	**(9) 692-4747**
Ambulance	**(9) 742-3300**	**(9) 692-4747**
Poison	**(9) 542-2323**	**(9) 542-2323**
Police	**(9) 911**	**(9) 549-8800**
Safety-Security	**Extension 8870**	

Emergency Room **Huntington Hospital** 270 Park Avenue, Huntington	**631-351-2300** **(1037)**
Dentists Dr. William Berg Dr. Robert Zeman	 **631-271-2310** **631-271-8090**
Doctor MediCenter 234 W. Jericho Tpke., Huntington Station	**631-423-5400** (**1034**)
Drugs - 24 hours, 7 days Rite-Aid 391 W. Main Street, Huntington	**631-549-9400** (**1039**)

Free Speed Dial
Dial the four numbers (********) from any **tan house phone** to place a free call.

GENERAL INFORMATION

Books, Gifts, Snacks, Clothing, Newspapers
BOOKSTORE 367-8837 (hours posted on door)
Located in Grace Auditorium, lower level.

Photocopiers, Journals, Periodicals, Books, Newspapers
Photocopying – Main Library
Hours: 8:00 a.m. – 9:00 p.m. Mon-Fri
10:00 a.m. – 6:00 p.m. Saturday
Helpful tips - Obtain PIN from Meetings & Courses Office to enter Library after hours. See Library staff for photocopier code.

Computers, E-mail, Internet access
Grace Auditorium
Upper level: E-mail only
Lower level: Word processing and printing.
STMP server address: mail.optonline.net
To access your E-mail, you must know the name of your home server.

Dining, Bar
Blackford Hall
Breakfast 7:30–9:00, Lunch 11:30–1:30, Dinner 5:30–7:00
Bar 5:00 p.m. until late
Helpful tip - If there is a line at the upper dining area, try the lower dining room

Messages, Mail, Faxes

Message Board, Grace, lower level

Swimming, Tennis, Jogging, Hiking

June–Sept. Lifeguard on duty at the beach. 12:00 noon–6:00 p.m. Two tennis courts open daily.

Russell Fitness Center

Dolan Hall, west wing, lower level
PIN#: **Press 64360 (then enter #)**

Concierge

On duty daily at Meetings & Courses Office.
After hours – From tan house phones, dial x8870 for assistance

Pay Phones, House Phones

Grace, lower level; Cabin Complex; Blackford Hall; Dolan Hall, foyer

CSHL's Green Campus

Cold Spring Harbor Laboratory is pledged to operate in an environmentally responsible fashion wherever possible. In the past, we have removed underground oil tanks, remediated asbestos in historic buildings, and taken substantial measures to ensure the pristine quality of the waters of the harbor. Water used for irrigation comes from natural springs and wells on the property itself. Lawns, trees, and planting beds are managed organically whenever possible. And trees are planted to replace those felled for construction projects.

Two areas in which the Laboratory has focused recent efforts have been those of waste management and energy conservation. The Laboratory currently recycles most waste. Scrap metal, electronics, construction debris, batteries, fluorescent light bulbs, toner cartridges, and waste oil are all recycled. For general waste, the Laboratory uses a "single stream waste management" system, removing recyclable materials and sending the remaining combustible trash to a cogeneration plant where it is burned to provide electricity, an approach considered among the most energy efficient, while providing a high yield of recyclable materials.

Equal attention has been paid to energy conservation. Most lighting fixtures have been replaced with high efficiency fluorescent fixtures, and thousands of incandescent bulbs throughout campus have been replaced with compact fluorescents. The Laboratory has also embarked on a project that will replace all building management systems on campus, reducing heating and cooling costs by as much as twenty-five per cent.

Cold Spring Harbor Laboratory continues to explore new ways in which we can reduce our environmental footprint, including encouraging our visitors and employees to use reusable containers, conserve energy, and suggest areas in which the Laboratory's efforts can be improved. This book, for example, is printed on recycled paper.

1-800 Access Numbers

AT&T	**9-1-800-321-0288**
MCI	**9-1-800-674-7000**

Local Interest

Fish Hatchery	631-692-6768
Sagamore Hill	516-922-4447
Whaling Museum	631-367-3418
Heckscher Museum	631-351-3250
CSHL DNA Learning Center	x 5170

New York City

Helpful tip -

Take Syosset Taxi to Syosset Train Station ($8.00 per person, 15 minute ride), then catch Long Island Railroad to Penn Station (33rd Street & 7th Avenue). Train ride about one hour.

TRANSPORTATION

Limo, Taxi

Syosset Limousine	516-364-9681 (**1031**)
Super Shuttle	800-957-4533 (**1033**)
To head west of CSHL - Syosset train station	
Syosset Taxi	516-921-2141 (**1030**)
To head east of CSHL - Huntington Village	
Orange & White Taxi	631-271-3600 (**1032**)
Executive Limo	631-696-8000 (**1047**)

Trains

Long Island Rail Road	822-LIRR
Schedules available from the Meetings & Courses Office.	
Amtrak	800-872-7245
MetroNorth	800-638-7646
New Jersey Transit	201-762-5100

Ferries

Bridgeport / Port Jefferson	631-473-0286 **(1036)**
Orient Point/ New London	631-323-2525 **(1038)**

Car Rentals

Avis	631-271-9300
Enterprise	631-424-8300
Hertz	631-427-6106

Airlines

American	800-433-7300
America West	800-237-9292
British Airways	800-247-9297
Continental	800-525-0280
Delta	800-221-1212
Japan Airlines	800-525-3663
Jet Blue	800-538-2583
KLM	800-374-7747
Lufthansa	800-645-3880
Northwest	800-225-2525
United	800-241-6522
US Airways	800-428-4322