DEFINITION OF VPU SENSITIVITY USING A MODEL VPU TARGET AND ROLE OF HYDROPHOBICITY OF THE MEMBRANE SPANNING DOMAIN IN THE VIRAL ENVELOPE GLYCOPROTEIN FUSOGENICITY

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

DEFINITION OF VPU SENSITIVITY USING A MODEL VPU TARGET AND ROLE OF HYDROPHOBICITY OF THE MEMBRANE SPANNING DOMAIN IN THE VIRAL ENVELOPE GLYCOPROTEIN FUSOGENICITY

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Dedication

"And, when you want something, the entire universe conspires in helping you to achieve it"- Paulo Coelho, The Alchemist.

"There is no religion higher than truth"- Motto of the Theosophical Society

I would like to dedicate my work to Truth and Knowledge which stands on the pillars of Hope, Logic, Hard-work and Determination.

Acknowledgement

I thank the motivations that allowed me to do a little more every day-a little more than I could have possibly done.

I thank my parents who have stood by my side and supported through my lofty jumps to reach my dreams. The character that holds me to my goals, the ideals that I measure myself by, awareness of reality that holds me to the ground are plants that they sowed and nurtured.

I thank the countless books and authors that have shaped my thoughts, the thoughts that preserved my sanity and made me who I am today, the lessons I learned from friends and others alike.

I thank Terri Lyddon for her support, help, affection and warmth. I shall remember your demands for “Palak Paneer” forever.

I thank the lab undergraduate students, who have made the lab a very pleasant atmosphere to work in. I thank Devon Gregory and Tiffany Lucas for their guidance. In the brief time I have discussed science with Daniel Salamango and Yul Eum Song, I thank them for their respect.

I thank my Mentor, Dr. Marc Johnson for his invaluable help with all that I am today. Thank you for being the complementary cog that turned my unoiled mental gears. Thank you for helping me acheive my goals, for helping me put my thoughts into a cohesive whole. Thank you for your hands-on mentoring, and the invaluable lessons that I shall take with me to a future in science.

Preface

The work outlined in this dissertation is the result of my effort in the Department of Molecular Microbiology and Immunology under the direction of Dr. Marc Johnson at the Christopher S Bond Life Sciences Center, University of Missouri, Columbia. This work has not been submitted to any other Academic institution for any reason. This dissertation explains work solely performed by me, unless otherwise cited. Work done by others has been appropriately cited and mentioned within the body of the document. Collaborative work with others has also been appropriately mentioned.

This work has been performed to understand the nature of glycoprotein recruitment by retroviral particles. Studies geared towards understanding the mechanism of glycoprotein recruitment to viral particles could potentially allow us to optimize this recruitment process, providing a cure against HIV. Further, optimization of glycoprotein acquisition could help design effective retroviral vectors for gene therapy.

The following research articles are a part of this dissertation

- **Janaka SK**, Devon Gregory, Johnson MC, "Hydrophobicity of membrane spanning domain of Env dictates fusogenicity, but not Env recruitment to viral particles", Manuscript in preparation.
- **Janaka SK**, Faurot J, Johnson MC, "Functional complementation of defective gamma-retroviral envelopes to study Vpu sensitivity of Gibbon ape Leukemia Virus envelope cytoplasmic tail domain", Manuscript under review; Submitted to PLoS One.

- Lucas TM*, **Janaka SK***, Stephens EB, Johnson MC, "Vpu down modulates two distinct targets, tetherin and Gibbon ape Leukemia Virus envelope, through shared features in the Vpu cytoplasmic tail", PLoS ONE. 2012. 7(12): e51741. * These authors contributed equally to this work.

- **Janaka SK**, Lucas TM, Johnson MC, "Sequences in Gibbon ape Leukemia Virus envelope that confer sensitivity to HIV-1 accessory protein Vpu", J. Virol., 2011 Nov;85(22):11945-54

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Abstract

Retroviral compatibility with diverse glycoproteins has been known and identified through the course of several studies. However, molecular mechanisms of glycoprotein acquisition are poorly defined. Glycoproteins are acquired by the virus as it buds out of the cell at the plasma membrane. Budding of retroviruses involves multilple interactions between viral and cellular proteins and a mature viral particle is the consummation of a regulated and a sequential process. Currently there are no drugs to target the assembly step of retrovirus.

In the series of studies outlined here, we outline a physical factor, Vpu that contributes to glycoprotein exclusion from HIV particles. Using a model Vpu target, Gibbon ape Leukemia Virus (GaLV) Env, we have deduced the characteristics of a protein that is targeted by Vpu through its cytoplasmic tail domain (CTD). This unique observation of Vpu modulating the GaLV Env CTD allowed us to compare the two modes of Vpu mediated protein modulation- CTD mediated and membrane spanning domain (MSD) mediated.

Subsequently, we studied the contribution of MSD hydrophobicity to Env recruitment to viral budding sites. Curiously, although hydrophobicity of MSD did not dictate Env recruitment, the helicity changes as a result of our mutations resulted in observation of the Env fusogenicity.

I. Introduction

A virus as a physical and functional entity requires the close co-operation of several cellular and viral proteins. The genome and the structural proteins assemble specifically with other viral components from the same or related viruses. In the case of enveloped viruses, it is known that foreign glycoproteins can assemble with different viral cores to produce infectious particles. Such a property is of tremendous interest in the field of gene therapy. Acquisition of foreign glycoproteins in addition to the native viral glycoproteins provides an opportunity to target different cells and tissues using similar viral vectors. Targeted gene delivery and specific regulation of genes in a tissue- specific manner would pave way for curing congenital diseases and certain infectious diseases in the long run. In particular, retroviruses have been used in such a context to cure patients with severe combined immunodeficiency (SCID). Foreign glycoproteins have been incorporated into replication incompetent retroviral vectors in order to achieve gene transfer to cells that are not targeted by the viruses in the natural course of infection. Such a tool needs to be understood completely, and the individual components have to be optimized, so that safe, effective and efficient gene delivery can be achieved. Retroviral vectors, having been clinically proven and well studied, provide an excellent model to undertake studies of vector optimization. Several components of retroviruses have been characterized, and diverse retroviral vectors for routine use in different applications have been developed. However, the mechanism of incorporation of glycoproteins into retroviruses is not completely understood.

Retroviruses

A retrovirus is an enveloped single stranded, dimeric RNA virus. They use the enzyme Reverse Transcriptase (RT) to produce a dsDNA version of the RNA genome and also integrate into the host cell genome using the Integrase (IN) protein. A common feature of the retroviruses is the presence of Gag, Pol and Envelope (Env) genes (Figure 1-1).Within the retroviral family, there are Alpha-, Beta-, Gamma-, Delta-, Epsilon-, Lenti- and Spuma- retroviruses (Figure 1-2). Gag is the structural protein, while Pol provides enzymatic functions. Env produces the surface glycoprotein that helps in targeting the virus to a specific cell type. The enzyme RT allows reverse transcription of the viral genome and is the defining characteristic of retroviruses. Retroviruses are integrated into the host cell genome at the end of an infection process. Para-retroviruses are a family of viruses that encode RT and reverse transcribe their genome, but do not integrate into the host cell genome. Simple retroviruses like the Alpha, Beta, Gamma, Epsilon sub families are made up of only the aforementioned three basic genes, whereas complex retroviruses like the Lenti, Delta and Spuma retroviruses have accessory proteins that help in viral pathogenesis (Figure 1-3). The accessory proteins provide vital functions and help in replication and/ or pathogenecity of virus. While not all accessory proteins are essential for replication of the respective virus, some of them are instrumental in the virus overcoming host restriction factors.

Figure 1-1: **Retroviral genome schematic and structure of a retrovirus**

Retroviral genome encodes for Gag, Pol and Env proteins with the terminal repeats(R) and is common to all retroviruses. The genomic RNA is capped on its 5' end and has a poly-A tail on its 3' end, just like cellular mRNA. Other features in the RNA genome are shown; PBS-Primer binding site; PPT-Poly purine tract; Ψ-Packaging element. Untranslated 5' and 3' regions are marked as U5 and U3 respectively. Structure of a mature retrovirus is also shown. Image indicates the structural proteins from Gag, Functional enzymes from Pol, Envelope proteins as hetrodimers and the presence of dimeric RNA. Image from Hubpages (http://enni82.hubpages.com/hub/Overview-of-Retrovirus-Life-Cycle#)

Figure 1-1: Retroviral genome schematic and structure of a retrovirus

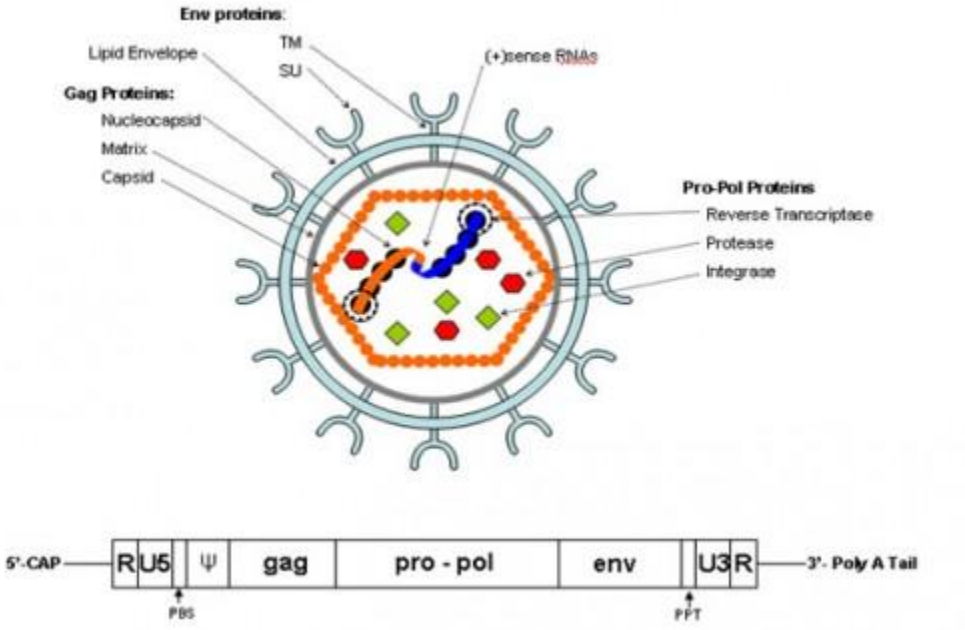


Figure 1-2: **Phylogeny of retroviruses**

Unrooted phylogenetic tree depicting the various retroviral sub-families are shown. The distinction between simple and complex retroviruses is made on the basis of absence or presence of accessory proteins. Some viruses belonging to the different sub-families are indicated. Superscript red stars indicate simple retroviruses. Image from (233).

Figure 1-2: Phylogeny of retroviruses

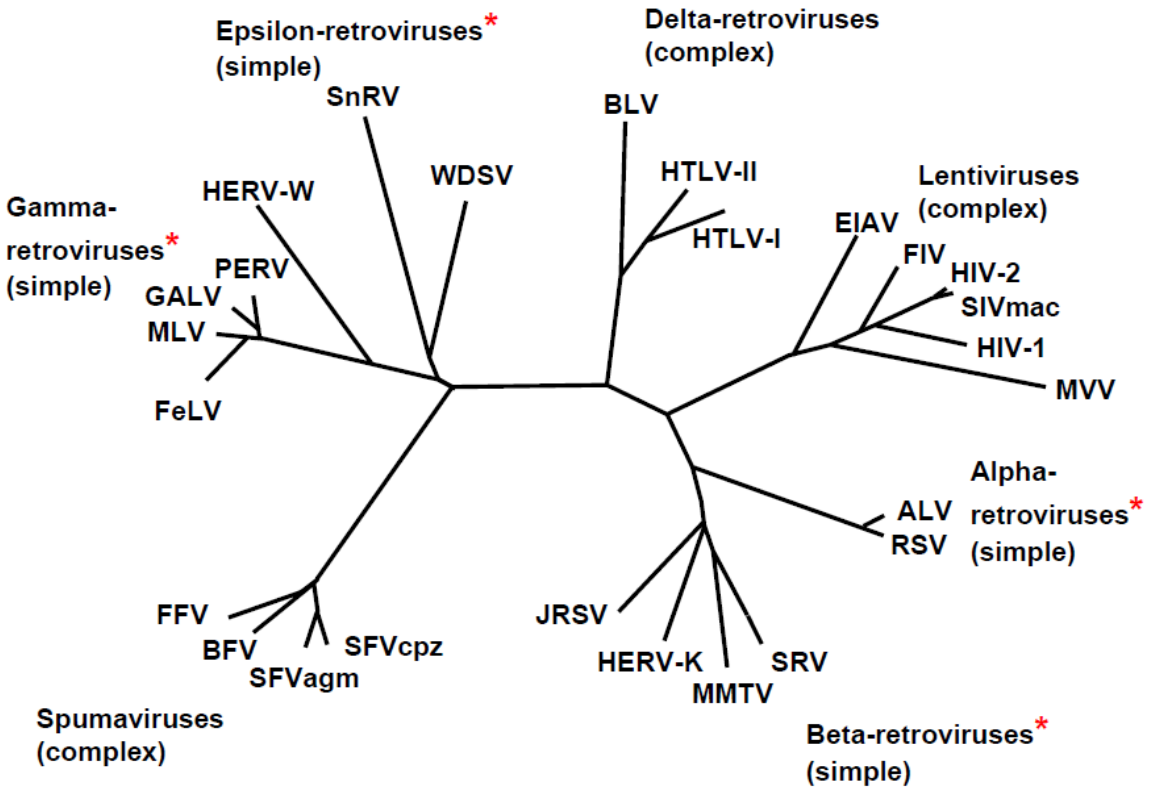
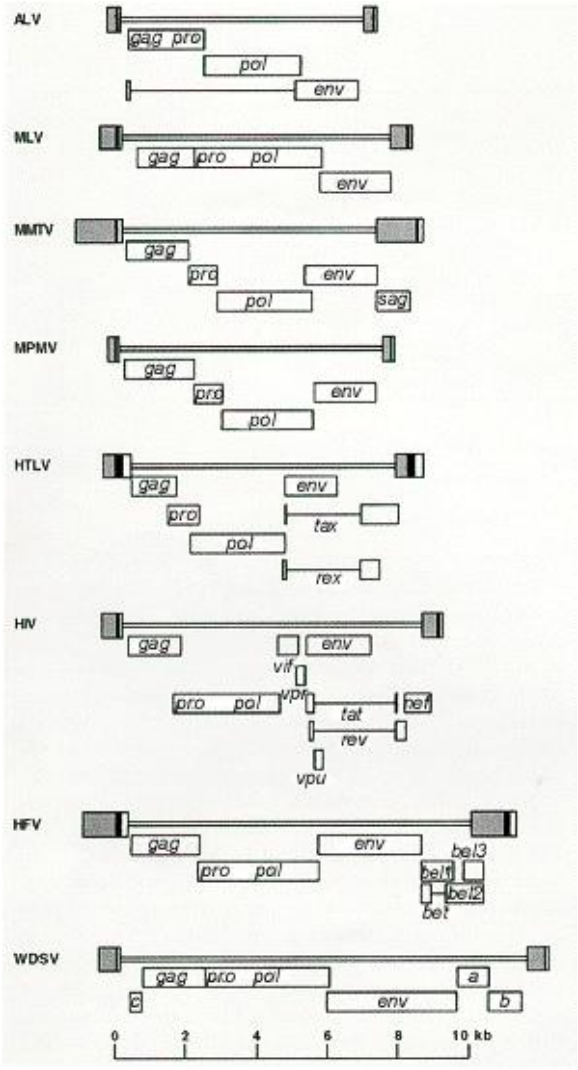


Figure 1-3: **Genetic organization of prototypic retroviruses**

Figure shows the different genes encoded by the genomes of the indicated viruses, representing all the classes of retroviruses shown in Figure 1-2. Simple retroviruses encode only Gag, Pol and Env. Complex retroviruses encode other accessory proteins in its genome. Image adapted from (286).

Figure 1-3: Genetic organization of prototypic retroviruses



Retroviral Proteins and Genome

Among retroviruses, HIV-1 is very well studied and characterized. HIV-1 Gag is produced as a single 55 kDa protein encompassing the N-terminal Matrix (MA), Capsid (CA) and C-terminal Nucleocapsid (NC) domains. Gag acts as the structural component of the virus. In all retroviruses, the N- terminal MA is post-translationally myristoylated at the glycine in position 2. Myristoylation is critical for membrane binding (228). HIV-1 MA possesses a PIP2 (Phosphatidylinositol-4, 5-bisphosphate) recognition motif that also sereves to anchor the protein to the membrane by interaction with PIP2. MA can also bind to nucleic acids and may determine the binding of MA to the membrane. A highly basic region (HBR) in the MA composed of several lysines helps bind the MA to the negatively charged lipid head groups in the membrane (54, 105). MA may also bind to nucleic acids to prevent non-specific binding to lipid head groups (7). Typical retroviral structures are made possible by CA (6). HIV-1 CA has two domains-N terminal domain (NTD) and C terminal domain (CTD) that are joined by a flexible linker. NTD forms hexameric and pentameric rings, while the CTD forms homodimers that allows the capsid to assume the core structure (18). The C- terminal NC domain recognizes and interacts with the viral genomic RNA during viral assembly. Also, NC provides crucial interactions among HIV-1 Gag molecules in the immature particle. Presence of Gag alone can promote the production of virus like particles (VLP), in the presence or absence of other cellular factors. Gag is also known to be phosphorylated, although the functional relevance of this modification is not understood yet (4, 18, 77, 272).

Generally in retroviruses, the Gag ORF is transcribed and translated into the Gag protein only. At times, a ribosomal frameshift occurs, resulting in the translation of Gag-Pol (277). In the case of Murine Leukemia Virus (MLV), the Gag-Pol is produced by read-through or suppression of the stop codon. In Beta- and Delta-retroviruses, protease is encoded in an ORF independent of Gag and Pol. In such cases, two frameshifting events occur, to ensure the production of Gag-Pro-Pol. With Spumaviruses, the Pro-Pol is produced independently of Gag (232) (Figure 1-4).

The Pol encodes the Protease (PR), Reverse transcriptase (RT), and Integrase (IN) proteins. Pol is the functional enzymatic unit of the virus. PR cleaves itself out of the Pol protein and proteolytically releases the individual structural and functional domains from the Gag and Pol proteins. PR activation happens at or immediately after the point of viral release from the host cell and this process is termed as viral maturation. RT helps in converting the genomic RNA into cDNA. IN helps in integrating the viral dsDNA, produced by RT, into the target cell genome (286).

The viral RNA genome is flanked by repeats (R), untranslated 3' region (U3) preceding R at the 3' end of genome and untranslated 5' region (U5) succeeding R at the 5' end of the genome. In the integrated proviral DNA form, U3-R-U5 forms the long terminal repeats (LTR) at either end of the genome (Figure 1-5). The LTRs possess promoter activity and help in transcription of the viral encoded between the two LTRs. Certain cis acting elements like the primer binding site (PBS), packaging element (Ψ),

dimerization inititation site (DIS) and poly purine tract (PPT) are absolutely required for producing infectious particles. Ψ is recognized by NC and determines packaging of the genome into the viral particle. Regardless of the genome size, a RNA dimer is packaged into the virus and genomic RNA dimers are created by interaction of RNA molecules at the DIS. The structure of the HIV-1 genomic RNA has recently been deduced (292) (Figure 1-6). In addition to the genomic RNA and the tRNA primer, cellular RNA is also packaged into a budding viral particle (286).

Retroviral Env glycoproteins deck the surface of the virus. Env provides the functions of receptor recognition and fusion with host cell membrane. Env is produced from a spliced genomic RNA, which lacks Gag and Pol coding regions. Env is trimeric and is composed of heterodimers containing a surface subunit (SU) and a transmembrane subunit (TM) (Figure 1-7). The N-terminal portion of the SU dictates the binding of the virus to a specific surface receptor protein on the target cell. The SU is linked to the TM by covalent or non-covalent interactions. SU and TM are held together by an inter-subunit disulphide bond in the case of alpha-, gamma-, and delta-retroviruses (80, 122, 152, 208, 223, 225); and non-covalently associated in the case of lenti- and beta-retroviruses (102, 143). Binding of the SU to its cognate receptor triggers a conformational change in the Env, leading to coreceptor binding or the exposure of the fusion peptide at the N-terminus of TM, which acts like a class I fusion protein (108, 286).

Env undergoes multiple changes during its biogenesis and trafficking in the virion producing cell. The Env is produced as a type I transmembrane precursor and upon signal peptide recognition, is translated from ER bound ribosomes (Figure 1-8). The signal peptide is co-translationally cleaved. The Env is also glycosylated co-translationally. N-linked glycosylation occurs on the asparagine in the canonical N-X-S/T motif, where X is any amino acid but proline or glycine. O-linked glycosylation also occurs on the Env. The range of glycosylation varies in different retroviral Envs and within a host due to the high mutation rate of retroviruses several quasispecies with different sites of glycosylation are present. Env trimerizes in the ER and this process is thought to be the rate limiting step in Env synthesis (108, 286). Env glycoproteins with similar ectodomains may heterotrimerize and this has been demonstrated with HIV-1and HIV-2 Env (41). Post ER, the precursor undergoes furin cleavage to produce the mature Env possessing the SU and TM domains. The ectodomain of TM and the SU are then linked covalently by cysteine disulphide linkage(s) or non-covalently. The furin cleavage is essential for fusogenicity and infectivity with Env (83-84, 168, 184). With MLV Env, when cleavage is blocked higher molecular weight species of Env have been observed, and that may represent an intermediate in the biogenetic pathway(84).

Lentiviral glycoproteins have a very long cytoplasmic tail domain (CTD) compared to those of other retroviruses (Figure 1-9). The length of the CTD in lentiviruses may represent an evolutionary significance (227). Such an idea is supported by the fact that, truncation of the CTD in causes multiple changes in Env trafficking, virus incorporation, fusogenicity, cell surface expression etc. in a cell type dependent

manner (58, 227). In the specific case of Feline immunodeficiency virus (FIV) Env, truncation of the entire CTD enhances incorporation into viral particles, while partial truncations in the CTD affect fusogenecity and surface expression (40). Tyrosine and di-leucine motifs in HIV-1 Env CTD are thought to interact with adaptor-protein complexes, that also interact with the respective Gag proteins (14, 67). A combination of such trafficking motifs allows an optimal level of HIV-1 Env surface expression that prevents a high level of immune response and at the same time allows efficient HIV-1 virion production. The YxxL tyrosine motif is also known to target HIV-1 Env to the cellular basolateral surface in polarized epithelial cells, cell-cell transmission in T-cells and efficient particle infectivity (64, 161-162). The Yxxϕ tyrosine motif (where ϕ is any hydrophobic amino acid) in MLV Env or HTLV Env also dictates trafficking, as mutations within this motif affects Env distribution (64).

Retroviral glycoproteins are palmitoylated or possess consensus sequences recognized by the cellular palmitoylation machinery (101, 204, 307). Palmitic acid is a fatty acid modification of membrane bound proteins, usually by a thio-ester linkage to cysteine residues at or close to the membrane. The hydrophobicity of the palimitic acid helps the protein bind to the membrane. Mutation of palmitoylation sites in HIV-1 Env, MLV Env or RSV Env results in decreased association with lipid rafts, endocytosis, virion incorporation, surface expression, but no apparent decrease in fusogenicity (20, 158, 204, 242). Palmitate thus provides multiple benefits to the Env proteins- in terms of incorporation by virtue of surface expression and association with lipid rafts, where Gag has been shown to assemble.

Beta- and Gamma-retroviral Envs possess an R-peptide in their C-terminus (32, 100). The R-peptide is cleaved by the viral protease to activate the Env for fusion. Presence of R-peptide is fusion inhibiting, as shown with the Influenza virus Hemaglutinin protein (157). Env fusogenicity is dependent on receptor binding followed by conformational changes in the heterodimeric Env protein (59, 165, 290).

Figure 1-4: **Gag, Pol translational mechanisms**

Expression of Gag and Gag-Pol occurs from translation of the same mRNA. Translation past the 3′end of the Gag-coding sequence results in the synthesis of Gag-Pol fusion proteins. Gag-Pol translation occurs by ribosome framseshifting (fs), or suppression of stop codon (is). Organization of the various retroviral genomes depicted shows the production of Gag-Pol in the different retroviruses (232).

Figure 1-4: Gag, Pol translational mechanisms

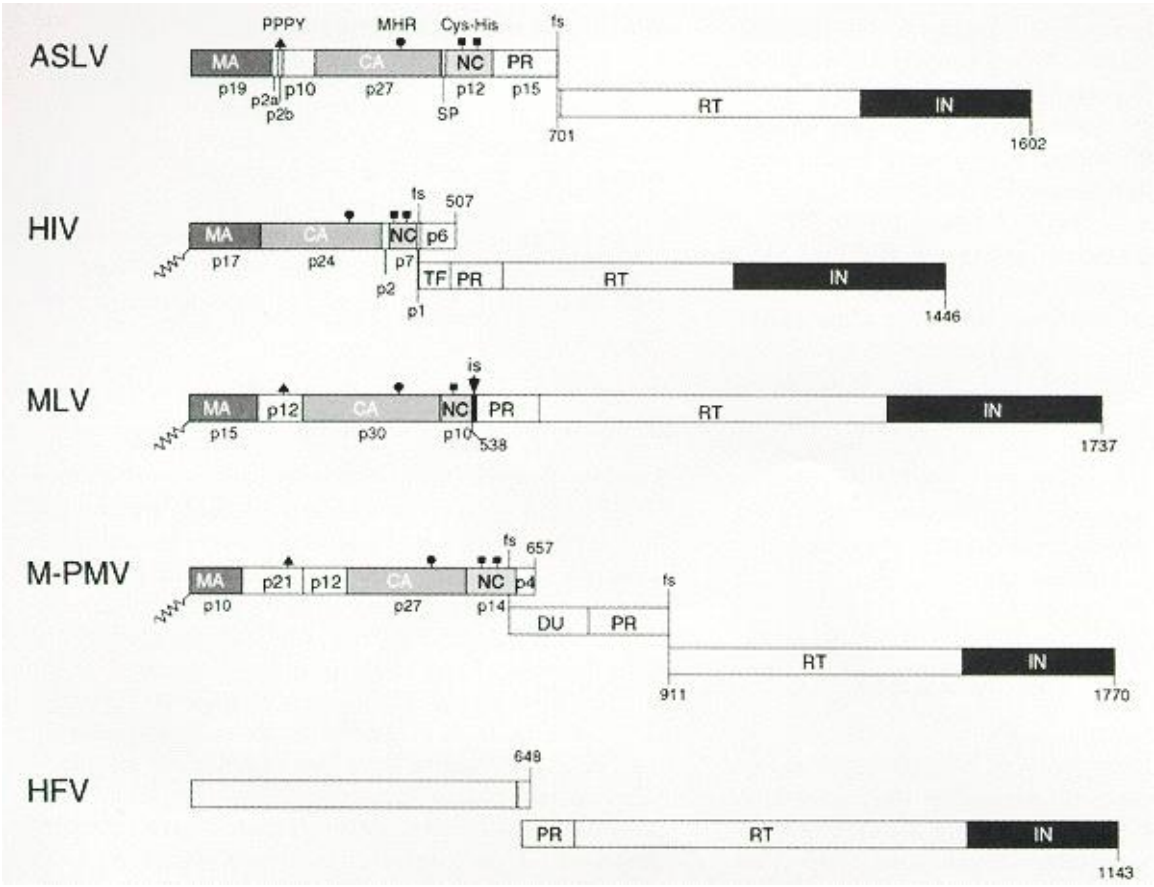


Figure 1-5: **Host cell integrated, proviral DNA and genomic RNA**

Figure indicates the organization of the DNA form of the integrated provirus, showing the different genes encoded within the genome. The DNA form is flanked by long terminal repeats (LTRs) characterized by the U3-R-U5 on either end of the genome. The RNA form shows the transcribed genes with a splicing intermediate, that allows translation of viral proteins in the infected cells. The RNA is similar to cellular mRNA and differs from the proviral DNA form by the absence of LTRs. Image from (286).

Figure 1-5: Host cell integrated, proviral DNA and genomic RNA

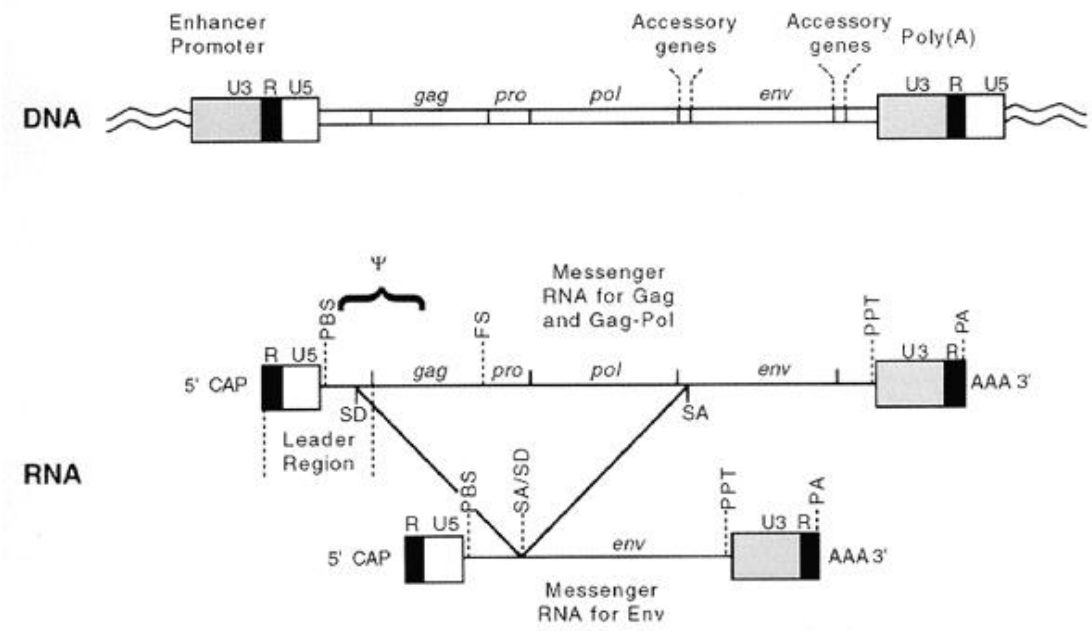


Figure 1-6: **Structure of HIV-1 RNA**

Figure depicts the structure of HIV-1 NL4-3 RNA as determined by Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE). The various features of the genomic RNA are labeled (292).

Figure 1-6: Structure of HIV-1 RNA

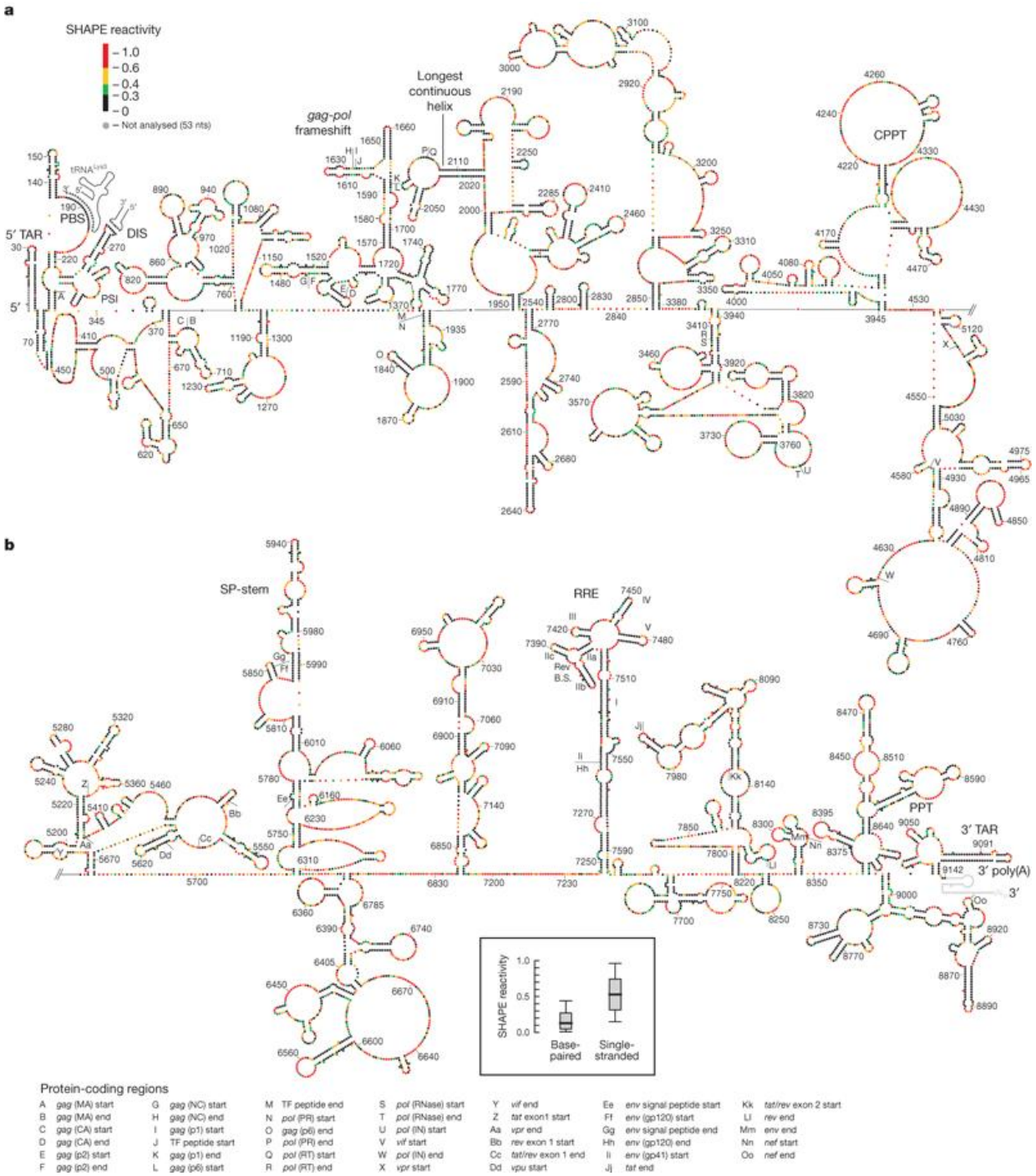


Figure 1-7: **Cartoon representation of Env trimer**

Env consists of extracellular SU subunit and a transmembrane TM subunit. Various features of an Env are shown in this figure. RBD- Receptor binding domain; PRR- Proline rich region; HR- Heptad repeat; H8- Histidine 8; FP- Fusion peptide. Image adapted from (265).

Figure 1-7: Cartoon representation of Env trimer

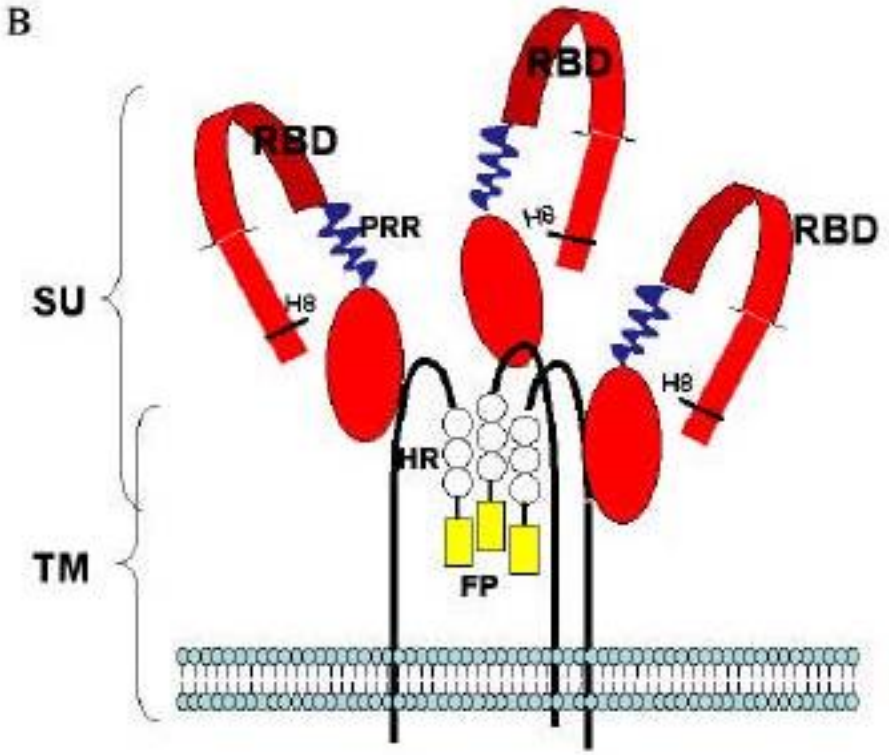


Figure 1-8: **Env biogenesis and trafficking**

Env is synthesized in the ER, where the signal peptide is cleaved and Env trimerizes. In the Golgi, Env is cleaved by furin or furin like protease into its constituent subunits. Env is then trafficked to the plasma membrane, where it gets incorporated into viral particles. Image from (273).

Figure 1-8: Env biogenesis and trafficking

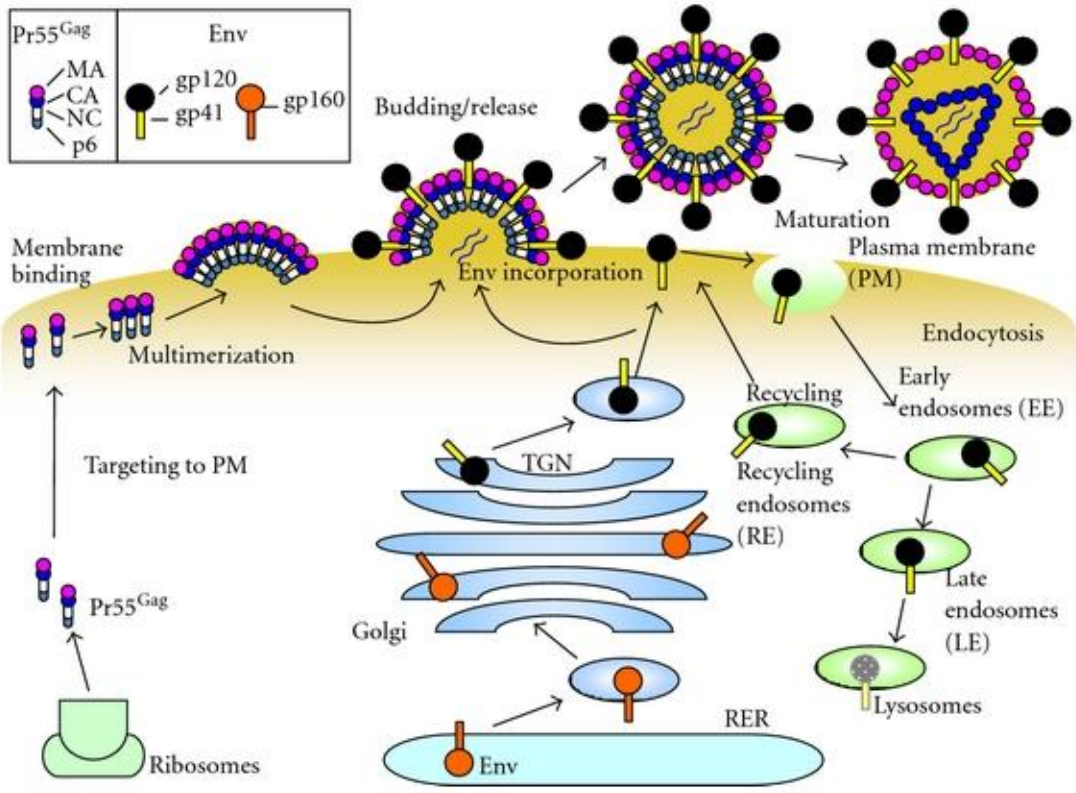
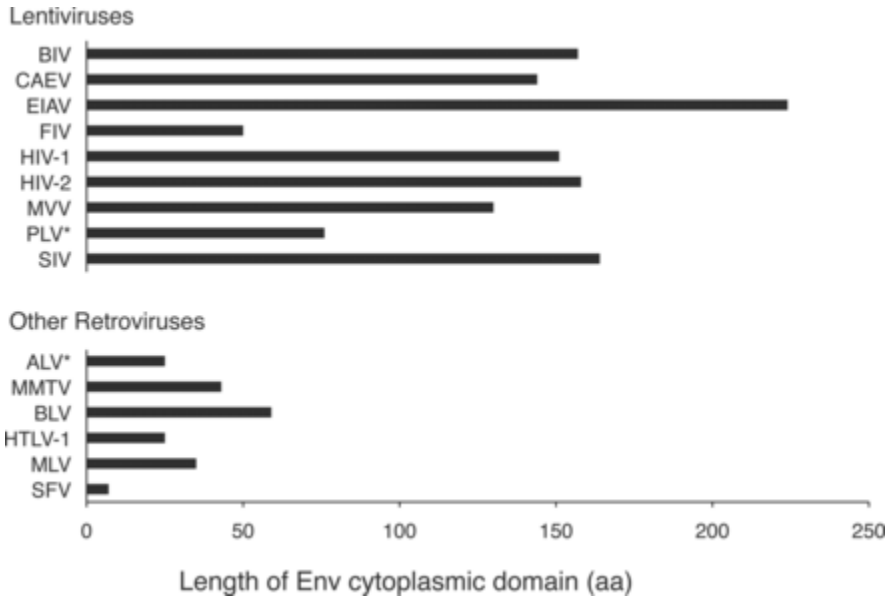


Figure 1-9: **Cytoplasmic tail length varies in different retroviruses**

Lentiviruses have much longer CTDs than other retroviruses. CTD influences incorporation of Env into retroviral particles and CTD lengths seem to be evolutionarily significant. Image from (227).

Figure 1-9: Cytoplasmic tail length varies in different retroviruses



Retroviral assembly

Assembly of an infectious retroviral particle requires that the RNA genome, Gag and Gag-Pol proteins and Env glycoproteins coalesce in the cell. Gag alone is capable of assembling into particles in cells and *in vitro*. To describe in broad terms, Gag interacts with genomic RNA and traffics the RNA to the membrane where other Gag moieties multimerize. This is followed by Env recruitment to the assembly site, resulting in budding of a complete virion with the help of other cellular factors (18, 232).

Retroviruses may assemble at the plasma membrane or in the cytoplasm as Intracellular Cytoplasmic A Particles (ICAPs). The former, termed as C-type assembly occurs by Gag multimerization at the plasma membrane and subsequent acquisition of glycoproteins prior to budding. The latter is termed as B/D type assembly and here Gag containing particles are formed in the cytoplasm, and the virions acquire the glycoprotein when they are trafficked to the membrane and exuded from the cell. Although distinct patterns of assembly exist, a single mutation in MPMV Gag switches the assembly phenotype from B/D type to a C-type assembly, indicating that assembly mechanisms might be similar (237). Many immature particles have been observed in the ER as Intracisternal A-type Particles (IAPs). These predominantly occur with endogenous retroviruses that encode defective components of Pol and Env (147) (Figure 1-10). While immature particles of all retroviruses have a spherical core with an electron-translucent center, mature particles have a specific morphology with an electron dense center and the

morphology is defined by the CA-CA interactions. CA cores have been shown to determine the size of the mature viral particles (6).

Gag is synthesized in the cytoplasm on free ribosomes. Multiple components in the virus interact sequentially to form an immature particle in the cell. Although the sequential order of the various interactions are not completely understood, retroviral assembly requires protein-protein, protein-RNA and protein-lipid interactions to produce an infectious particle. Using HIV-1 as a model, recently it has been shown that Gag interacts with the genomic RNA in the cytoplasm and forms lower order multimers in the cytoplasm, while higher order Gag multimers have been observed on membranes (149) (Figure 1-11). As mentioned earlier, Gag binding to membrane is strengthened by three different methods- (1) Interaction with the negatively charged lipid head groups through the HBR. (2) PIP2 interaction with MA on the inner leaflet of the membrane and (3) Myristate group based anchoring of MA to the lipid bilayer (34, 53, 61-62, 170, 197, 205). Recent reports of Rous Sarcoma Virus (RSV) Gag have shown that PIP2 does not influence membrane association (44) and compensatory mutations in HIV-1 Gag have been identified in MA when PIP2 in the infected cells is depleted (192). In direct contrast to such reports, HIV and MLV particles are enriched in PIP2 (45). It is not clear whether PIP2 influences the site of viral budding at the plasma membrane, or alternatively, if Gag allows an enrichment of PIP2 at the viral budding site.

The site of HIV-1 particle assembly is debated, with imaging studies showing the assembly process to occur on the inner leaflet of the plasma membrane (1-2, 111, 133)

(Figure 1-11). But, in certain cell types like macrophages, it has been shown to assemble in intracellular vesicles (63, 218). Later these vesicles were shown to be membrane invaginations that were continuous with the plasma membrane (19, 294). Spread of HIV-1 infection occurs more efficiently in a cell-to-cell manner, through interfaces of cell contacts called virological synapses, than by infection of fresh cells by cell-free virus (37, 127, 229, 258). Gag and Env coalesce at the virological synapse, in addition to other proteins like CD4, adhesion molecules etc (128, 229). MLV and HIV particles readily form the synapse, where Env receptor and other cell-cell interactions take place (185) (258) (123-126, 128, 315).

Lipid analysis of retroviral particles has shown the enrichment of lipid-raft associated lipds or lipids arranged in a lower mobility state in viral particles (221, 230-231, 266-267). Further, HIV-1 envelope is enriched for cholesterol and sphingolipds, found in lipid ordered microdomains. Also, cholesterol depletion in virus producing cells or viruses interferes with viral release, structural stability of virus, and infectivity (9, 33, 38-39, 90, 94). Cholesterol and other sphingolipids are found in higher concentrations in lipid- rafts, a lipid ordered microdomain, which has lower mobility of lipids within the raft region. Glycoproteins involved in signaling, phosphoinositides, etc. are enriched in the lipid rafts. Experimentally, lipid rafts are defined as detergent-insoluble membranes at 4^{o}C. Additionally, certain lipid-raft associated proteins have been reported to be incorporated to HIV-1 particles (202, 210) (245), while some non-raft proteins are excluded from viral particles (202). Gag association with drug resistant membranes supports the idea of HIV-1 budding from lipid rafts (66, 96, 106, 159, 202, 206-207,

222). At the moment, the role of lipid microdomains in the assembly and release of virus is not entirely clear, despite the multitude of studies performed on the lipid composition of viral particles and biochemical analysis of Lipid- Gag interaction (287-288).

While membranes may have several different 'microdomains' containing different lipid or protein compositions, HIV-1 assembly and replication have been most associated with lipid rafts and Tetraspanin enriched microdomains (TEMs). Tetraspanins, as the name suggests, contain four membrane spanning domains and are usually palmitoylated. HIV-1 is enriched in certain tetraspanins when they are expressed in high quantities in an infected cell. Tetraspanins seem to regulate HIV-1 Env fusogenicity and they colocalize with ESCRT1 components, which have been shown to facilitate HIV-1 budding. However, tetraspanins do not enhance or affect the budding process itself (144, 171).

The roles of lipids and tetraspanins in viral budding have not been clearly understood. Membranes are a fluidic body and the plausibility of static microdomains to which Gag binds is questionable. Although, it is entirely possible that Gag changes the local environment in the membrane. Gag multimerization may affect lipid and protein distribution at the virion assembly site, which in turn determines the constitutents of the budded viral particle. Virion associated host cell proteins are largely dependent on the cell producing the virus. Some cellular factors maybe specific to certain cell types and they may influence protein recruitment to budding viral particles, while certain other proteins maybe abundant in certain cell types and hence may be incorporated in virions (210).

In addition to Gag forming the viral particles, maturation of the budded virion requires the presence of PR and completion of the infection requires Pol. Covalent linkage of the Gag and Pol proteins ensures the recruitment of Pol into the Gag nucleated viral particles. However this translational linkage is not absolutely necessary, as accessory proteins like Vpr in HIV-1 is recruited to budding viruses, by virtue of its interaction with MA. Also, with spumaviruses, Pol protein is produced independent of Gag and is also incorporated efficiently into the viral particle. This suggests that although covalent linkage is a simple mechanism of protein incorporation in viral particles, the linkage is not absolutely essential. Many other cellular proteins are also incorporated into viral particles, by virtue of their abundance in the cytoplasm.

HIV budding has been shown to be dependent on the ESCRT-I complex and recruitment of Tsg101 belonging to the ESCRT complex is a result of interaction with HIV-1 Gag PTAP motif, termed as the late domain. MLV, RSV, HTLV-I, MPMV Gags possess a PPXY late domain motif, that may indirectly activate the ESCRT complex and thus help viral egress. Late domain mediated viral release may interact directly or indirectly with components of the ESCRT protein complexes. Mutation of the late domain mutants results in an immature particle phenotype (25-26, 55, 89, 99, 136, 180, 186, 216, 257, 301, 305, 309, 311).

Figure 1-10: **Different patterns of retroviral particle assembly**

B/D type of assembly occurs in intracellular locations, followed by trafficking of the particle to the plasma membrane where the Env is incorporated into particles. C-type assembly occurs on the inner leaflet of the plasma membrane, where Env trimers coalesce with the virion. Intracytoplasmic A-type particles (ICAPs) assemble in the lumen of ER. They remain unprocessed and without Env. Image from (232).

Figure 1-10: Different patterns of retroviral particle assembly

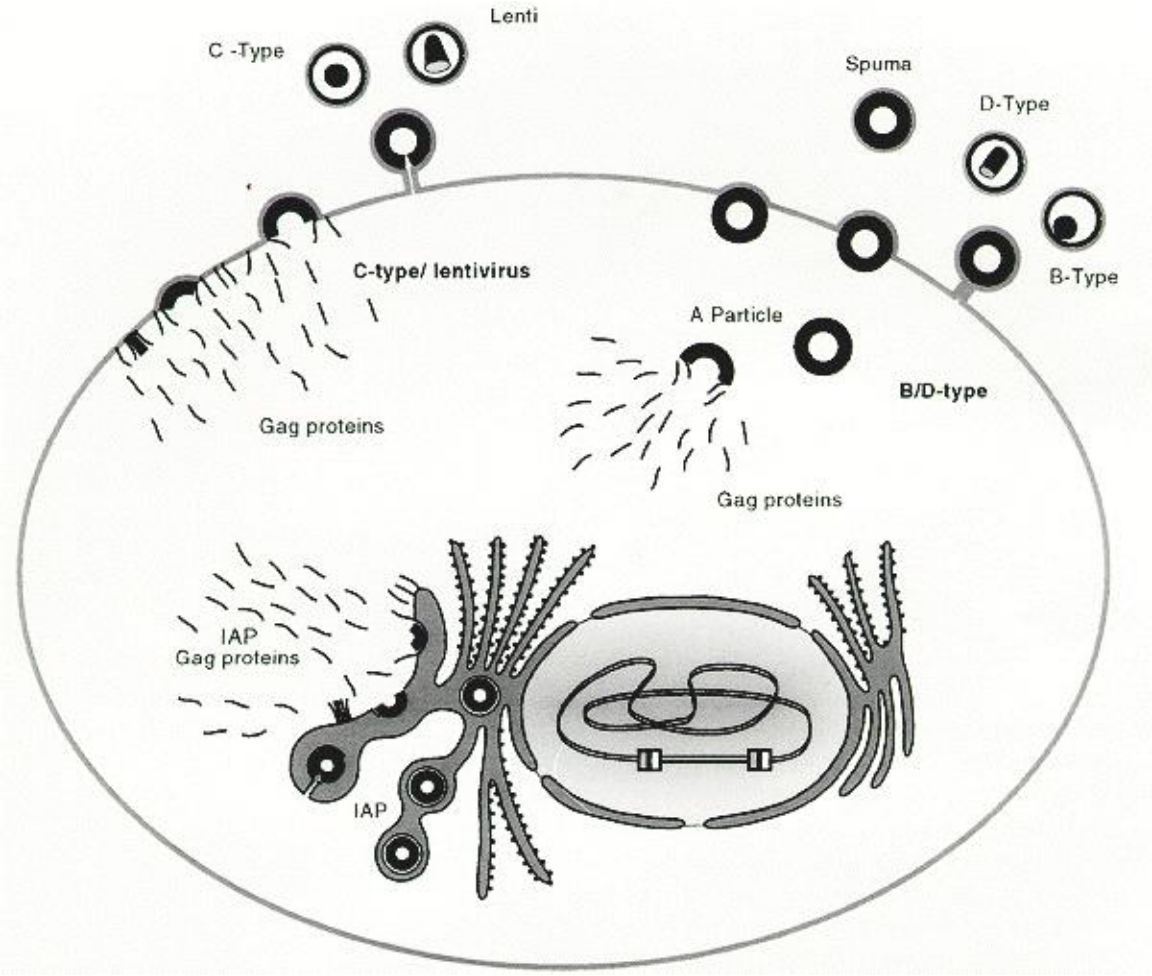
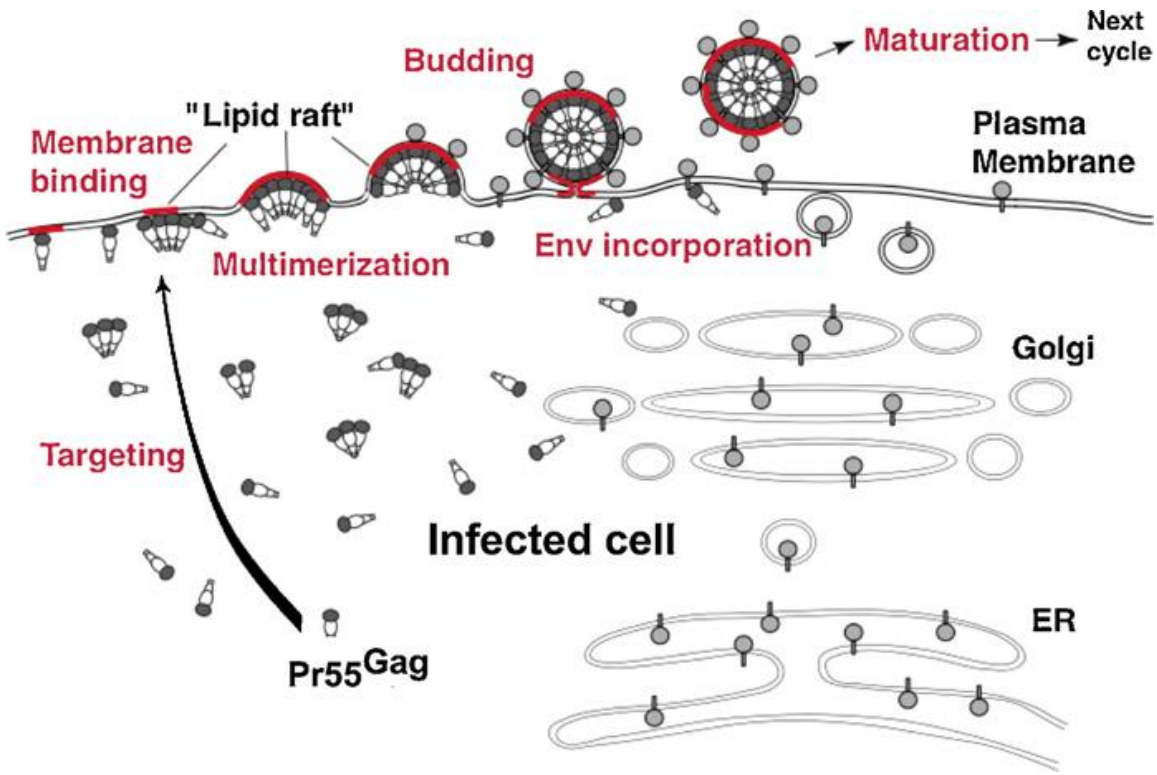


Figure 1-11: **C-type assembly of HIV-1 particles**

Lower order multimers of Gag in the cytoplasm traffic to the plasma membrane where, higher order multimers are formed. Lipid raft binding has been depicted in this cartoon. Env is recruited to viral budding sites and the virus buds, followed by maturation of virus. Image from (287).

Figure 1-11: C-type assembly of HIV-1 particles



Recruitment of Env to viral particles

Retroviruses have the ability to form infectious particles with foreign Env glycoproteins from different retroviruses and different viral families. This process is termed pseudotyping and it serves to change the tissue tropism of the respective viral particles (60, 121, 182, 214). Acquisition of the native Env may be a result of evolutionary selection and optimization. However, recruitment of cellular and foreign viral glycorproteins is surprising. Nonetheless, this provides a way of targeting similar vector particles to different tissues for application in gene therapy scenarios. While Gag is produced in the cytoplasm on free ribosomes, Env is produced on ER bound ribosomes. To be incorporated into virions, Env has to be trafficked to the membrane through which retroviral particles bud. The specific mechanisms of Env recruitment and trafficking are not clearly understood. All retroviral glycoproteins are produced as type I transmembrane proteins and later processed into a mature form, as mentioned earlier.

Some lentiviral glycoproteins exhibit incompatibility with non-native Gags, presumably due to steric incompatibility of Gag lattices with the long CTDs of the lentiviral Envs. With HIV-1 and SIV Env, the CTD has been shown to regulate viral replication, by either changing the site of budding in cells or by providing selective advantage for the virus in a cell dependent manner. In replication permissive cell lines, HIV-1 ΔCTD Env is incorporated into viruses at close to normal levels. In peripheral blood mononuclear cells and monocyte derived macrophages, HIV-1 ΔCTD Env is not incorporated into viruses efficiently and as a result these cell lines are non-permissive for

viral replication with ΔCTD Env. The ΔCTD truncation mutant by itself is processed well and is fusogenically active (5, 71, 86, 195, 295, 310). Although these differences among cell lines are not understood, it is clear that the CTD of HIV-1 Env dictates Env incorporation into virions (227). Conversely with SIV Env, CTD truncations spontaneously arise when passaged in human cells and results in better Env incorporation and higher fusogenicity. However, in Rhesus cells, SIV selects for Env with full length CTD. Such cell type dependence of the CTD of these Envs is not very well understood (42, 141-142). In the case of MLV Env, removal of the CTD does not prevent specific recruitment of Env to viral budding sites. However, in a competitive assay between HIV and MLV particles, the CTD dictates specific incorporation of Env into MLV particles (91, 168).

In MDCK cells, in the presence of HIV-1 Env, HIV viral assembly occurs on the basolateral surface and may be a direct result of enrichment of negatively charged phospholipid headgroups (64, 161-162). With rat neuronal cells, HIV-1 Env induces a redistribution of HIV Gag and MLV Env induces a redistribution of MLV Gag molecules (293). Also, MLV Env directs MLV Gag recruitment to viral budding sites in the virological synapse (315). These data indicate an Env directed assembly phenotype for retroviral particles. Further, non-native Envs are specifically incorporated into viral particles as shown with a correlative high-resolution imaging technique (131). MLV Env expression in the absence of Gag shows a random distribution on the surface of the cell. When MLV Env is expressed along with MLV Gag or HIV Gag, the Env on the cell surface is redistributed specifically around the viral budding sites (168). Collectively,

multiple studies on different Envs indicate a role of the CTD in recruitment to viral budding sites.

The tyrosine-sorting motifs in Env CTD play a role in Env directed virus assembly and pathogenesis in addition to regulating trafficking of Env. In polarized epithelial cells, the tyrosine motif determines basolateral targeting of Env and infectious viral budding. The tyrosine motif also allows the Env and Gag to coalesce at the virological synapse, helping cell-to-cell transmission of virus (64, 119, 161-162, 260). Another unique sorting motif found in the gammaretroviral RD114 Env causes incompatibility with SIV Gag. PACS-1, a cellular protein mimics SIV Gag in redistributing RD114 Env to endosomes by interaction with this acidic motif (27).

Another example of a cellular protein that mediates coalescence of HIV-1 Env with budding HIV virions is Tail Interacting Protein of 47kDa (TIP47), also known as Perilipin3. In the cell, TIP47 is implicated in lipid biogenesis and protein sorting to such lipids. TIP47 was reported to be a necessary connector between HIV MA and HIV Env CTD (17, 24, 163). Since then the role of TIP47 in Env recruitment has come into question with *in vitro* studies showing non-requirement of TIP47 for HIV MA and HIV Env CTD binding, and RNA mediated knockdown of TIP47 showing no change in infectivity, virion release and Env incorporation into HIV particles (47).

Retroviral Life Cycle

The budding virion, having thus assembled and acquired Env glycoproteins, is an infectious unit that may now infect a new target cell and may proceed with its life cycle (Figure 1-12). The receptor binding and/or coreceptor binding is followed by fusion of the viral and target cell membranes with the help of a type I fusion mechanism involving the fusion peptide in the Env (120). The viral components are then released into the host cell. The mature gag composing the core contains the enzymatic components along with the viral RNA. Reverse transcription of the viral genome takes place in the cytoplasm of the target cell. The exact order of the early events in an infection is not clear, with the timing of reverse transcription, capsid uncoating and nuclear import of the viral genome unresolved. tRNA molecules from the virus producing cell usually prime cDNA synthesis in retroviruses and they bind to the viral genomic RNA at the PBS. Different RNA/DNA intermediates are produced during the viral cDNA production by RT. PPT helps in priming the second strand synthesis in the dsDNA. RNaseH domain of the RT degrades the RNA in the RNA-DNA duplex intermediates during cDNA synthesis (3) (Figure 1-13). Reverse transcription can occur in the absence of the retroviral gene ORFs within the genome and this provides a basis for retroviral vectors.

The pre-integration complex (PIC) containing viral and some cellular proteins is imported into the nucleus (as in the case of lentiviruses) or accesses the host cell genomic content during cell division, when the nuclear membrane breaks down. Specific target sequences in the host cell DNA are not required for integration. IN helps in integrating

the dsDNA into the target cell genome (226). The different viral proteins are synthesized in the infected cell and the proteins are trafficked to the budding site. Unspliced genomic RNA is also recruited to the viral budding site by an interaction with NC. Env glycoproteins then coalesce at the budding viral particle. The viruses are then budded out of the cell at which point the PR is activated, mediating the maturation of the virus (57).

Figure 1-12: **Retroviral life cycle**

Cell-free virus binds to the receptor on the target cell surface and fusion of host and viral membranes occur. The contents of the viral particles are released into the cytoplasm of the target cell and reverse transcription of the viral genome takes place. The viral dsDNA is translocated to the nucleus as a part of the PIC. IN helps in integration of the viral DNA into the host cell genome. Viral proteins are synthesized; genomic RNA and other viral proteins are trafficked to the plasma membrane where, the viruses assemble and bud. Image from NCI, Open access.

Figure 1-12: Retroviral life cycle

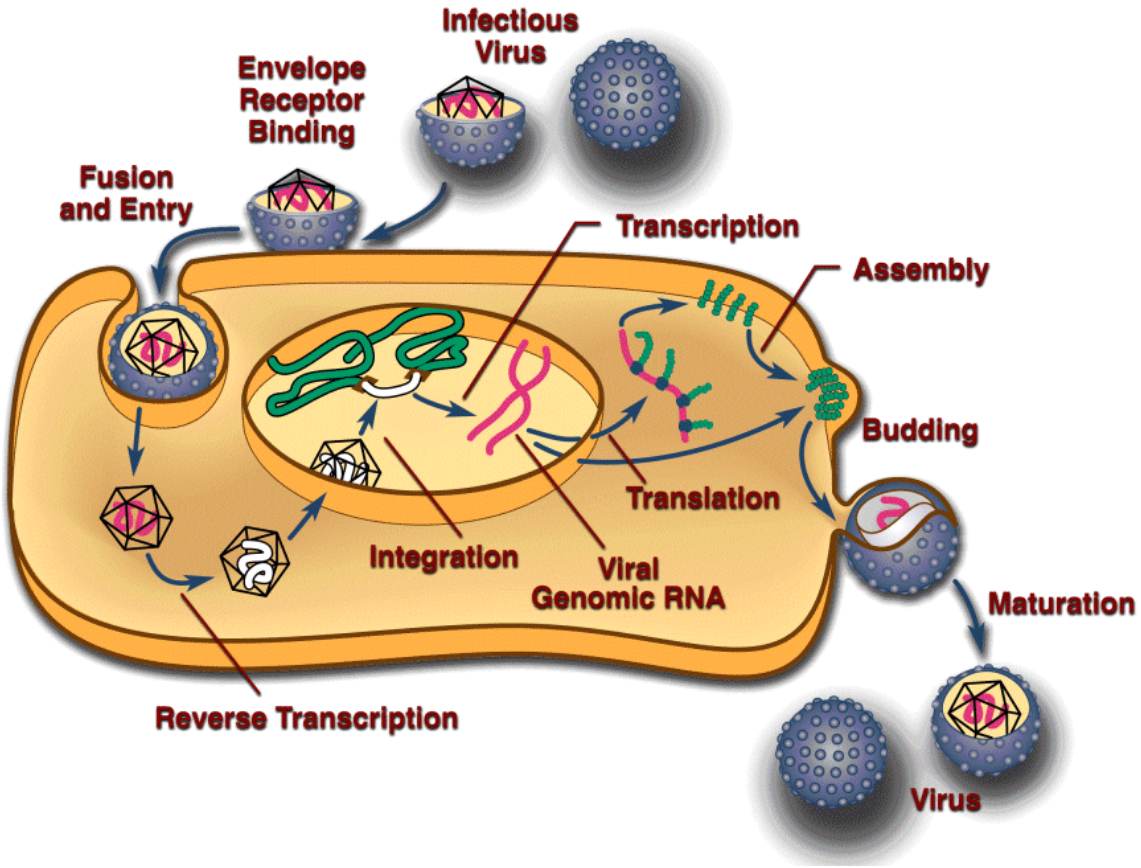
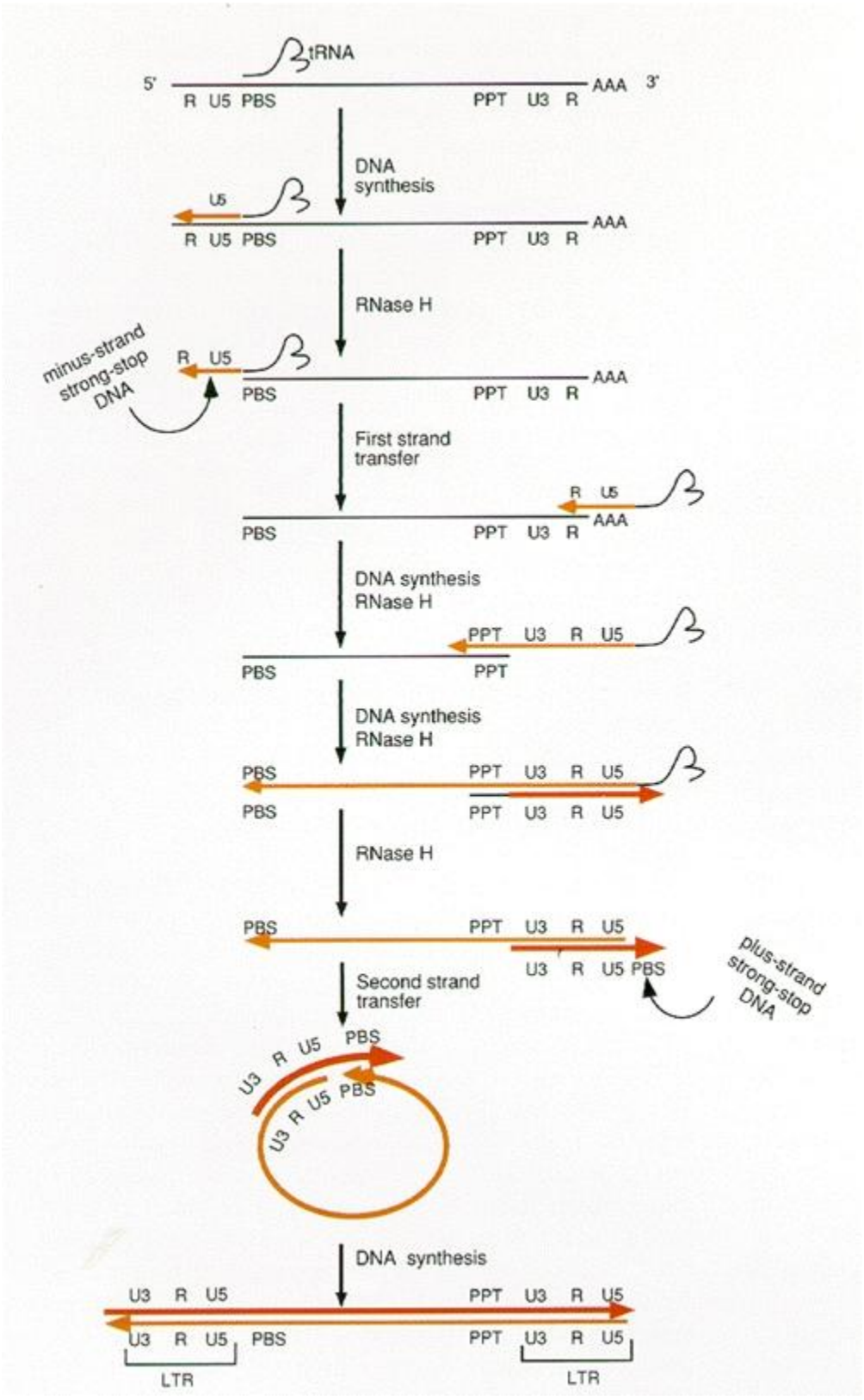


Figure 1-13: **Process of reverse transcription**

The different stages of reverse transcription of viral DNA are shown. Image from (3).

Figure 1-13: Process of reverse transcription



Retroviral Vectors

The Gag-Pol and Env may be provided in *trans* to the genomic element to produce infectious viral particles. This allows for the retroviral genes between the LTR to be replaced with a target gene of interest, conserving the necessary *cis* acting elements. This also serves the purpose of providing a larger piece of DNA that can be inserted between the LTRs. Since vectors used in gene transfer have to be non- replicative, such separation of the individual components helps in creating an effective vector. In fact, during the production of viral vectors, the *Gag*, *Pol* and the *Env* genes are removed from the genome to prevent replicative spread of virus and viral particles are produced by co-transfection of the individual encoding constructs. Further, several refinements of the vectors have led to the advent of self inactivating lentiviral and retroviral vectors, carrying chimeric LTRs (11, 137). Retroviral vectors have been successfully used in a clinical setting to treat patients with severe combined immunodeficiency syndrome (SCID) (23, 87). Several improvements have been made with retroviral vectors and the latest series of self-inactivating vectors will go a long way in gene therapy treatment without further complication from replication competent retrovirus (RCR). While retroviral vectors maybe used for targeted gene delivery to dividing cells, lentiviral vectors provide the unique opportunity to target terminally divided cells.

Env binds very specifically to its cognate receptor, and since different receptor proteins are expressed at different levels in various tissues, targeted gene delivery to different tissues cannot be achieved with the same vector. The vectors can be modified at

this juncture by providing a compatible Env from a different virus. The process by which viral particles of one type are produced with a glycoprotein from another virus is called pseudotyping and the viral particles so produced are called pseudotypes.

Pseudotyping of retroviral particles

Retroviral pseudotyping provides some obvious benefits in the targeted gene delivery module. On the one hand, viral tropism can be altered and the viral vector can be directed to different tissues and cell types that are not normally infected by the parent vector; On the other hand, use of lentiviral vectors with different Env proteins on the surface would enable gene delivery to different non- dividing cells, while other retroviral vectors can infect only dividing cells. Furthermore, stable integration and long term expression of any gene of interest can be achieved with retroviral vectors (11, 214).

Pseudotyping has been achieved within the retroviral family and also between different viral families. For example, Human Immunodeficiency virus (HIV), a lentivirus, is compatible with glycoproteins from many families of viruses, including alpha-, beta-, gamma-, delta-, spuma-retroviruses and other lentiviruses in addition to glycoproteins from rhabdoviruses, filoviruses, togaviruses, arenaviruses, coronaviruses, flaviviruses, orthomyxoviruses, paramyxoviruses and baculoviruses (50, 60, 104, 121, 139-140, 148, 150, 156, 160, 190, 193, 198, 214, 236, 312). The recruitment of native viral proteins to its budding sites in the cell maybe realized as a specific mechanism of viral survival.

Several lines of evidence point that native glycoproteins interact with the structural Gag protein. In the case of HIV, MA interacts with the cytoplasmic tail domain (CTD) of HIV Env and is required for incorporation of Env into viral particles (46, 194, 211). Also, the C-terminal 16 amino acids of MLV Env, termed the R-peptide, have been reported to be associated with the MLV viral core (10). Recruitment of a variety of foreign viral glycoproteins to viral egress sites, however, indicates a more generic property of viral envelope proteins. MLV Env and the G- protein from Vesicular stomatitis virus (VSV) are compatible with Rous sarcoma virus (RSV) cores and HIV cores (132). Some cellular transmembrane proteins are also incorporated into viral particles, potentially due to the high local concentration or random distribution of these proteins at the general area of viral budding (210). The viral components that promote recruitment of foreign glycoproteins and the physical factors that drive the Env towards other viral budding sites are not known. Comparison of mechanisms of pseudotyping and native glycoprotein acquisition will provide details of Env incorporation into several different viral particles.

Foreign Env acquisition by retroviral particles can be a result of passive and non-specific or active and specific incorporation. Pseudotyping of retroviral particles and cellular glycoprotein incorporation into viral particles supports the notion of a passive process. Specific recruitment of certain Env glycoproteins may also exist- (1) Convergent trafficking to membrane microdomains; (2) Direct interactions between viral structural proteins and Env; (3) Interaction with cellular proteins mediating coalescence. Several examples of specific recruitment of Env to particular viral particles have been reported. However, the molecular mechanisms of Env recruitment are not known and an over-

arching mechanism of glycoprotein recruitment to viral particle has not yet been postulated.

Pseudotyping of HIV particles with MLV Env

HIV is able to form infectious particles with the gammaretroviral MLV Env. By correlative SEM imaging, our lab has shown that MLV Env is recruited specifically to viral budding sites. Further, the CTD of MLV Env is not required for this specific recruitment (168). MLV Env possesses a fusion restricting R-peptide in its C-terminus that is removed by the viral protease during viral maturation. HIV PR is also able to cleave the MLV Env R-peptide, thus making the viral particle infectious (50, 91).

The studies detailed in the following chapters outline mutational analyses that add to the understanding of physical factors favoring glycoprotein recruitment to viral budding sites. A gammaretroviral Env like MLV Env or GaLV Env has been used as a model to study glycoprotein recruitment. The following two chapter outline sequences and characteristics of a model Vpu target protein, the Gibbon ape Leukemia Virus (GaLV) Env, that dictate its exclusion from HIV-1 particles (113, 115). In the fourth chapter, details of a study consisting of mutational analysis of the CTD of Vpu that was performed to identify sequences in Vpu that prevented the trafficking of GaLV Env and Tetherin (an antiviral, interferon induced host cell glycoprotein) to HIV-1 budding sites is described (166). The fifth chapter describes the study of the hydrophobic patch of amino acids in the MLV Env membrane spanning domain (MSD) (114). This study yielded interesting information about Env fusogenicity.

II. SEQUENCES IN GIBBON APE LEUKEMIA VIRUS ENVELOPE THAT CONFER SENSITIVITY TO HIV-1 ACCESSORY PROTEIN VPU

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Abstract:

HIV-1 efficiently forms pseudotyped particles with many gammaretrovirus glycoproteins such as Friend murine leukemia virus (F-MLV) Env, but not with the related gibbon ape leukemia virus (GaLV) Env or with a chimeric F-MLV Env with a GaLV cytoplasmic tail domain (CTD). This incompatibility is modulated by the HIV-1 accessory protein Vpu. Because the GaLV Env CTD does not resemble tetherin or CD4, the well studied targets of Vpu, we sought to characterize the modular sequence in the GaLV Env CTD required for this restriction in the presence of Vpu. Using a systematic mutagenesis scan, we determined the motif that makes GaLV Env sensitive to Vpu is INxxIxxVKxxVxRxK. This region in the CTD of GaLV Env is predicted to form a helix. Mutations in the CTD that would break this helix abolish sensitivity to Vpu. Although many of these positions can be replaced with amino acids with similar biophysical properties without disrupting the Vpu sensitivity, the final lysine residue is

required. This Vpu sensitivity sequence appears to be modular, as the unrelated Rous sarcoma virus (RSV) Env can be made Vpu sensitive by replacing its CTD with the GaLV Env CTD. In addition, F-MLV Env can be made Vpu sensitive by mutating two amino acids in its cytoplasmic tail to make it resemble more closely the Vpu sensitivity motif. Surprisingly, the core components of this Vpu sensitivity sequence are also present in the host surface protein CD4, which is also targeted by Vpu through its CTD.

Keywords: Vpu; GaLV; F-MLV; CD4; Vpu sensitivity motif; Envelope protein

Introduction:

Human immunodeficiency virus (HIV-1), like many viruses, is capable of assembling infectious viral particles using the surface glycoproteins from foreign viruses by a process termed pseudotyping. However, not all virus/glycoprotein pairs are able to complement one another. HIV-1 is compatible with glycoproteins from many families of viruses including rhabdoviruses, other retroviruses, and filoviruses, but the compatibility does not strictly follow family lines (135, 156, 214). For instance, HIV-1 is compatible with the glycoprotein from the gammaretrovirus Friend murine leukemia virus (F-MLV Env), but it is not compatible with the glycoprotein from gibbon ape leukemia virus (GaLV) Env, even though F-MLV and GaLV belong to the same genus (50, 69, 118). GaLV is a gammaretrovirus found in captive gibbon apes. It is closely related to a retrovirus found in wild koalas (koala retrovirus, KoRV), but both viruses are believed to be fairly recent introductions that likely were derived from endogenous mouse retroviruses [reviewed in (275)]**.** The F-MLV and GaLV Env glycoproteins display 48% identity at the amino acid level. Both proteins have a native molecular weight of ~85 kDa, and both are cleaved by a cellular protease into the 70 kDa surface (SU) and 15 kDa transmembrane (TM) domains, which remain associated after cleavage. Both the F-MLV and GaLV TM domains are additionally cleaved in their cytoplasmic tail domain (CTD) into a 12 kDa (p12E) and a 2 kDa peptide (p2, or R-peptide) by the virus-encoded protease during the viral assembly process (118, 224). This R-peptide cleavage is required for the viral glycoproteins to become fusogenically active (224). The component of GaLV Env that causes the incompatibility with HIV-1 has been mapped to its CTD (50, 269). Recently, we and others demonstrated that the incompatibility of HIV-1 with

glycoproteins containing the CTD from GaLV Env is dictated by the HIV-1 accessory protein Vpu (52, 167). In the presence of Vpu, GaLV Env CTD containing glycoproteins are prevented from being incorporated into HIV-1 particles; whereas, deletion of Vpu restores incorporation of these glycoproteins and infectivity of the resulting HIV-1 particles. The mechanism for this GaLV Env exclusion is not known, although it has been suggested that this may be affected by difference in trafficking of Env in the presence of Vpu (52).

Vpu is an 81 amino acid HIV-1 protein that contains an N-terminal membrane-spanning domain followed by an ~50 amino acid cytoplasmic tail (270). Vpu is unique to HIV-1 and a few closely related lentiviruses. The first and most widely studied function of Vpu is to promote the degradation of the host surface protein CD4, the primary receptor for HIV-1. Since HIV-1 Env can bind to CD4 during transit through the endoplasmic reticulum (ER), binding can result in the proteins being sequestered in the ER. This can result in severe impairment to viral propagation, and has been thought to be a major reason for Vpu's role in CD4 degradation (154, 239, 274). The C-terminal cytoplasmic domain of Vpu interacts with the CD4 cytoplasmic tail; consequently, the E3 ubiquitin ligase complex bearing β–TrCP is recruited to CD4 (179). CD4 is subsequently ubiquitinated and degraded by the proteasome (179, 238, 250). β–TrCP is critical for this function, and the phosphoserine residues in positions 52 and 56 of Vpu are required for β–TrCP recruitment and for CD4 degradation (255).

Vpu also enhances viral release by modulating the host defense protein tetherin (also known as BST-2, CD317 or HM1.24) (271, 281). Tetherin is an interferon-α induced anti-viral protein that contains an N-terminal membrane-spanning domain and a C-terminal glycophosphatidylinositol anchor that physically tethers enveloped viruses to the infected cell's surface after release. Human tetherin expression at the cell surface is efficiently modulated by Vpu, resulting in enhanced virus release (271, 281). Unlike with CD4, recognition of tetherin by Vpu appears to be facilitated through the membrane spanning domains of the two proteins (81, 220). Vpu with a scrambled membrane spanning domain cannot enhance viral release or modulate tetherin activity (217, 253, 278). Tetherin antagonism by Vpu has been reported to be β-TrCP dependent (117, 188) and has been reported to involve the sequestration of tetherin in the trans-Golgi network or the endo-lysosomal compartments (73). In addition to modulating tetherin and CD4, Vpu has also been reported to degrade or modulate other host cellular proteins, including TASK-1, MHC-I, MHC-II, CD1d and NTB-A (107, 109, 135, 191, 259).

GaLV and HIV-1 infect different primates and belong to different genera of retroviruses. The reason for the antagonism of GaLV Env by HIV-1 Vpu is not clear. We hypothesize that the proteins containing the GaLV Env CTD resemble human proteins that are normally targeted by Vpu and are mistakenly recognized by Vpu and excluded from viral particles. The possibility that the resemblance between GaLV Env and the natural target of Vpu has a functional significance cannot be excluded. An alanine-scanning mutagenesis strategy combined with a single-round infectivity assay performed

in the presence or absence of Vpu was performed to identify the precise amino acid sequence in the GaLV Env CTD that confers Vpu sensitivity. The primary protein sequence conferring sensitivity to Vpu was further validated by showing that additional viral glycoproteins could be made sensitive to Vpu by mutating select amino acid residues to match the identified Vpu-sensitivity sequence.

Materials and Methods

Plasmids

The ecotropic F-MLV Env (isolate 57) expression construct was kindly provided by Walther Mothes (Yale University). The F-MLV/GaLV chimeric Env construct containing the full-length form of GaLV Env CTD was constructed using oligonucleotide linkers coding for the cytoplasmic tail of GaLV. These oligonucleotides were inserted between the ClaI site (encoded within DRL amino acid coding sequence, 30 amino acids upstream of the C-terminus as shown in Figure 2-1), and the EcoRI site that occurs after the stop codon in the cytoplasmic tail region of the F-MLV Env expression construct. Plasmids expressing the truncated forms of the F-MLV/GaLV Env (i.e., lacking 4, 8, 9, 10, 11 or 12 amino acids at the C-terminus) were constructed by introducing a stop codon at the appropriate position on the linkers used to replace the fragment between ClaI and EcoRI. Mutant chimeric F-MLV/GaLV Env expressing constructs were made in the context of the Δ8 F-MLV/GaLV chimeric Env (lacking 8 amino acids at the C-terminus) and were constructed by site-directed mutagenesis of the fragment between ClaI and EcoRI. Two silent mutations were made from ‘ctcatt’ to ‘ctgatc’ to encode LII amino

acids 48 residues upstream of the C-terminus in the transmembrane region to introduce a BclI site into the region encoding the transmembrane domain of F-MLV Env. For mutations in the transmembrane region or the membrane proximal cytoplasmic domain of the chimeric F-MLV/GaLV Env, the fragment between BclI and EcoRI was replaced by PCR and the respective mutations were introduced through the primers. Order of the amino acid mutations indicated in this study is numbered from the C-terminus inward. Rous sarcoma virus Schmidt-Ruppin A (RSV) Env with GaLV CTD was created by a two-step PCR with oligonucleotides amplifying the sequences from the RSV Env transmembrane domain and ectodomain and the GaLV Env CTD. The fragment of DNA between EcoRI (encoding 'GIP') and SacII (after the stop codon) on the RSV Env expression plasmid was replaced by the PCR product. The RSV/GaLV Env fusion sequence was CLPC/ILNR. The C-terminal 27 amino acids in the vesicular stomatitis virus-G protein (VSV-G) were deleted, and the amino acids 'LRV' were silently mutated to introduce a unique MluI restriction site. The GaLV Env CTD, amplified by PCR, was introduced into the VSV-G encoding plasmid between MluI (encoding 'LRV') and BamHI (after the stop codon) sites to construct a VSV-G/GaLV chimeric Env. The VSV-G/GaLV Env fusion sequence was IHLCI/LNRLV. The influenza Hemagglutinin (HA) and Neuraminidase (NA) expression constructs pEWSN-HA and pCAGGS-WNA15, respectively, were kindly provided by Yoshihiro Kawaoka (University of Wisconsin-Madison). Influenza HA with GaLV Env CTD was created by a two-step PCR and replacement of the fragment between BstXI (encoding 'ASSLV') and XhoI (after the stop codon). The HA/GaLV Env fusion sequence was LGAIS/QFIND. The sequences of the inserted region in all the constructs were confirmed by sequencing. NL4-3 derived HIV-

CMV-GFP was kindly provided by Vineet KewalRamani (National Cancer Institute-Frederick). This proviral vector lacks the genes encoding Vif, Vpr, Vpu, Nef and Env and has a CMV immediate-early promoter driven GFP in the place of Nef. The Vpu^+ HIV-CMV-GFP was created by replacing the fragment between BamHI and SalI sites in HIV-CMV-GFP from the equivalent BamHI-SalI fragment encoding Vpu from the plasmid ΔR8.2 (316).

Cell culture

The 293FT cell line was obtained from Invitrogen. The cell line expressing the ecotropic F-MLV Env receptor, 293T mCAT-1, was kindly provided by Walther Mothes. The 293T TVA cell line expressing the receptor for Rous sarcoma virus (RSV) Env was provided by John Young (Scripps Research Institute) (156). All three cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418. In phases of cell culture involving transfection or transduction, G418 was not added to the medium.

Infectivity/Vpu sensitivity assays

Infectivity assays using HIV-CMV-GFP and its derivative were performed by transfection of 293FT cells with 500 ng of the HIV proviral DNA and 500 ng of the Env or the mutant Env expression construct using 3 μl of FuGENE 6 (Roche) or 3 μl of 1 mg/ml polyethylenimine (PEI) (31) in six well plates. When influenza HA or its derivatives were used, 293FT cells were transfected with 500 ng of the provirus, 250 ng of the HA expression vector or its derivative, and 250 ng of the NA expression vector.

The media were replaced 16 to 24 h post transfection in the case of FuGENE or 4 to 6 h post transfection in the case of PEI to remove any residual transfection reagent. Supernatant was collected 24 h after the media were exchanged and then frozen at -80^{o}C for at least 2 h to lyse any cells in the supernatant. The supernatant was thawed in a 37^{o}C water bath and spun at 2500 g for 10 min to pellet any cells or cell debris. For viruses pseudotyped with F-MLV Env or influenza HA, 1 ml of the supernatant was applied to fresh 293T mCAT-1 cells. For the transduction of RSV Env pseudotyped viruses, 1 ml of the supernatant was applied to fresh 293T TVA cells. Cells were collected 48 h later, fixed with 4% paraformaldehyde, and analyzed by flow cytometry using FACScan or Accuri C6 flow cytometer systems. Data were analyzed using FlowJo software (version 7.5.5, Tree Star). Vpu sensitivity is expressed as a ratio of the percentage of cells infected with the HIV-1 from provirus lacking Vpu to the percentage of cells infected with the HIV-1 from provirus containing Vpu (Figure 2-1B). Relative infectivity is expressed as a ratio of percentage of cells infected with Vpu^{-} HIV-1 pseudotyped with a mutant Env to the percentage of infectivity with Vpu^{-} HIV-1 pseudotyped with F-MLV Env in the same experiment (Figure 2-1B).

Western blotting

Transfections for western blots were performed as described for infectivity assays. Viral supernatants were spun at 13,200g for 2 h. The pellets were resuspended in 2X SDS-PAGE loading buffer and the equivalent of 1ml of viral supernatant was analyzed by 10% discontinuous SDS-PAGE. Cells were pelleted and resuspended in 1X SDS-PAGE loading buffer and approximately 2% of the total amount of cells was analyzed in parallel with the viral supernatants. Proteins were transferred manually onto a

0.22 μm PVDF membrane. The membranes were blocked with 5% nonfat dry milk and probed with goat anti-MLV Env gp70 (kindly provided by Alan Rein, NCI-Frederick) diluted 1:10000 and anti-HIV p24 hybridoma medium diluted 1:500 (obtained through the AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH: HIV-1 p24 hybridoma [183-H12-5C]) from Bruce Chesebro (49) and anti- HIV MA hybridoma medium diluted 1:5 (obtained through ATCC : HB-8975; MH-SVM33C9) (247). Blots were then probed with horse radish peroxidase conjugated anti-goat antibody diluted 1:10000 or anti-mouse antibody diluted 1:10000, both from Sigma. Luminata Crescendo western HRP substrate from Millipore was used for visualization of the membranes on a chemiluminescence image analyzer, LAS3000 from Fujifilm. Blots were also probed with IR dye 700DX anti mouse IgG and visualized using Odyssey infrared imaging system from LI-COR biosciences.

Results

F-MLV and GaLV are both gammaretroviruses, and the Env proteins of these viruses are closely related. The amino acids sequences of the TM domains of these two proteins are 65% identical and both proteins contain a conserved viral protease cleavage site that removes the C-terminal R-peptide. The R-peptide itself is less conserved than the rest of Env and differs in length by one amino acid (Figure 2-1A). In spite of these similarities, an F-MLV Env that contains the GaLV Env CTD is incompatible with HIV-1 proviruses that contain Vpu, while wild type F-MLV Env is compatible. This incompatibility typically causes a 50- to a 100-fold reduction in the number of infectious

HIV-1 particles produced (167). Vpu's action on GaLV Env appears mechanistically similar to its action on the host protein CD4. GaLV Env and CD4 contain short CTDs that dictate Vpu sensitivity. The modulation of CD4 and GaLV Env by Vpu is abolished by mutation of the serine residues at positions 52 and 56 in Vpu (167, 255), but it is not affected by mutations in the membrane-spanning domain of Vpu (167, 296). However, the CTD of GaLV Env is similar to the CTD of F-MLV but very dissimilar from the CTD of CD4 (Figure 2-1A), and yet GaLV Env but not F-MLV Env is sensitive to Vpu. In addition, while Vpu has typically been thought to target CD4 for degradation, the action on GaLV appears distinct. Vpu prevents GaLV Env CTD-containing F-MLV Env from forming infectious particles with HIV-1 but not with F-MLV (50-51, 167), which suggests that the mechanism of restriction is not based simply on protein degradation. These data suggest that the recognition sequence and modulation mechanism of GaLV Env by Vpu is distinct from that of CD4.

The boundary for Vpu sensitivity lies around the tenth amino acid from the C-terminus

To identify the precise sequence required for modulation of GaLV Env by Vpu, we first defined the C-terminal boundary that confers Vpu sensitivity. An F-MLV/GaLV Env chimera containing the C-terminal 29 amino acid CTD from GaLV Env was engineered to lack its C-terminal 4, 8, or 12 amino acids (denoted Δ4, Δ8, and Δ12) (Figure 2-2). The F-MLV/GaLV chimera was used so that the only differences between the Vpu-insensitive F-MLV Env and the Vpu-sensitive F-MLV/GaLV Env were in the

CTD. Infectivity experiments were performed in parallel with Vpu^+ and Vpu^- HIV proviruses using a 1:1 (wt:wt) ratio of proviral DNA to Env. This ratio of transfection yielded high infectivity and at least a 10-fold reduction in infectivity in the presence of Vpu with Env proteins that contained the GaLV CTD. A Vpu insensitive Env, F-MLV Env, was used as a control to ensure that observed differences were due to Vpu. In all cases, infectivity with F-MLV Env was essentially identical with the Vpu^+ and Vpu^- proviruses. The F-MLV/GaLV Env Δ4 and Δ8 proteins alone remained sensitive to Vpu; however, this sensitivity was abolished with the Δ12 truncation. Curiously, F-MLV/GaLV Env chimeras missing 8 to 12 amino acids at the C-terminus produced several fold more infectious particles than full-length F-MLV/GaLV Env (Figure 2-2B). Full length F-MLV/GaLV Env was incorporated into viral particles less efficiently than wildtype F-MLV Env, F-MLV/GaLV Env Δ8, or F-MLV/GaLV Env Δ12 (Figure 2-S1A). Reduced incorporation of full length GaLV Env into HIV particles, even in the absence of Vpu, has been noted previously (51). The reduction in incorporation also correlated with loss in infectivity with these mutant Envs, but did not affect Vpu sensitivity (Figure 2-2B, S1b). To further define the Vpu sensitivity boundary, F-MLV/GaLV chimeric Env proteins lacking 9, 10, or 11 amino acids from the C-terminus were created. The sensitivity toward Vpu was reduced stepwise with 9 or 10 amino acids being removed. Upon removal of 11 amino acids, the sensitivity toward Vpu was abolished (Figure 2-2A, B). Hence the boundary of the region sensitive to Vpu is the cytoplasmic tail extending to the Tyr at the tenth position from the C-terminus.

Vpu sensitivity motif

To identify the amino acids required for sensitivity toward Vpu, an alanine scanning mutagenesis was performed, starting from the minimal sensitive protein, F-MLV/GaLV Env Δ8. Initially, five amino acids at a time were mutated to alanine in the CTD of the chimeric Env. F-MLV/GaLV Env Δ8 containing mutations (from the C-terminus) 9-13A, 14-18A, and 24-28A formed infectious particles but were resistant to Vpu (Figure 2-S2). The construct with mutations 19-23A, however, did not form infectious particles in the presence or absence of Vpu. We fine tuned the mutagenesis by mutating only two amino acids at a time to alanine and observed that 11-12A, 13-14A, 15-16A, 17-18A, 19-20A, 21-22A, and 25-26A formed infectious particles but were not sensitive to Vpu. The exceptions were 23-24A and 27-28A, which did not produce infectious particles in the presence or absence of Vpu, and 9-10A, which produced infectious particles and remained sensitive to Vpu (Figure 2-S3).

Finally, the amino acids in the CTD of F-MLV/GaLV Env Δ8 were mutated individually. Of the 30 amino acids mutated, eight independently abolished sensitivity to Vpu. Mutants were scored as 'Vpu insensitive' if the presence of Vpu reduced the infectivity by less than four fold. The Vpu-sensitivity conveying residues were spread over a 16-residue segment beginning 26 amino acids from the C-terminus and conveyed Vpu sensitivity through the motif INxxIxxVKxLxxRxK (Figure 2-3). Mutation of amino acids V29, R31, N32, and L33 to alanine abolished the production of infectious particles with HIV-1 in the presence and absence of Vpu and therefore cannot be excluded as

being required for Vpu sensitivity. Several other point mutations altered the infectivity of particles produced, but there was no obvious correlation between changes that affect Vpu sensitivity and changes that affect infectivity (Figure 2-3). The 'sensitivity-motif' is composed largely of hydrophobic amino acids and a few positive charges but is devoid of proline and glycine residues, which is consistent with an alpha helical structure.

Modularity of the Vpu sensitivity motif-containing GaLV CTD

The GaLV Env CTD is required, but not necessarily sufficient, to confer Vpu sensitivity. Additional requirements for Vpu sensitivity may be present in F-MLV and GaLV Envs. To determine if this sensitivity is necessary and sufficient for mediating Vpu sensitivity, the CTD of RSV Env, HA, and VSV-G were replaced with the Vpu sensitivity motif from GaLV Env. Replacement of the CTDs of VSV-G and HA resulted in chimeric proteins unable to form infectious particles with HIV-1 in the presence or absence of Vpu (data not shown). However, replacement of the RSV CTD with the Vpu sensitivity motif from GaLV resulted in a chimera that produced infectious particles with HIV-1 and was sensitive to Vpu (Figure 2-4). Wildtype RSV Env could pseudotype HIV-1 particles efficiently and remained Vpu insensitive. Hence, sensitivity toward Vpu can be conferred on other proteins by exchanging the CTD with the GaLV Env CTD, but determination of whether the protein can form infectious particles depends on additional factors.

Sensitivity toward Vpu requires a putative alpha helix-

The frequency and positioning of hydrophobic amino acids in the GaLV Env CTD is consistent with an alpha helical domain. Secondary structure predictors indicate that the CTDs of F-MLV Env and GaLV Env are alpha helical with an unstructured chain of nine amino acids at the C-terminus (35, 130, 276). The putative alpha helix was disrupted to determine if this structure is important for Vpu sensitivity. Glycine and proline have a low propensity to fall into an alpha helix (212). Several point mutations were made throughout the Vpu-sensitivity sequence by changing individual amino acid residues to proline. Mutations of six different amino acids to proline in a stretch of 16 amino acids each abolished sensitivity toward Vpu (Figure 2-5B). In each case, HIV-1 particles pseudotyped with these mutant Envs displayed viral infectivity equivalent to the parent protein, suggesting the mutations did not alter normal protein function. These data are consistent with an alpha helix in the CTD of GaLV Env being required for Vpu sensitivity but not for regular infectivity function of Env in cell culture.

If the GaLV Env CTD is represented as an alpha helix, then all amino acids in the sensitivity motif, with the exception of R13, align on one side of the helix. Positions a and d in the helix contain largely hydrophobic amino acids and hence may be the interaction face of a coiled coil structure (Figure 2-5B). In fact, F-MLV Env is known to possess a trimeric structure in the ectodomain, and it is conceivable that the cytoplasmic domain also trimerizes with these hydrophobic amino acids providing the interaction for trimerization (276). The protease cleavage site for R-peptide removal is located at

position g of the helix and is free from the interaction face to be accessed by the viral protease to cleave the R-peptide and activate the Env (276). At position e of the helix is a positively charged face in the predicted alpha helix.

F-MLV Env can be made sensitive to Vpu

To validate the Vpu sensitivity motif, we tested whether Vpu sensitivity could be conferred to F-MLV Env with minimal changes. Although the F-MLV Env and GaLV Env CTDs differ at 12 residues out of 29 positions, only four of these positions are part of the predicted Vpu sensitivity motif (Figure 2-6A). Of these four, we focused on K11, K18, and N25, which are in the position e of the putative alpha helix (Figure 2-6B). The tail lengths of F-MLV and GaLV Env differ by one amino acid. N25, K18, and K11 in the GaLV Env from the C-terminus correspond to K26, Q19, and Q12 in the F-MLV Env from the C-terminus. These amino acids fall on the same face of the putative alpha helix. Q12K mutation alone conferred a slight sensitivity to Vpu upon F-MLV Env. However, mutations Q12K and Q19K were sufficient to make F-MLV Env acutely sensitive to Vpu. Q12K and K26N mutations, in contrast, were not sufficient to confer Vpu sensitivity (Figure 2-6C).

Vpu sensitivity requires lysine at the eleventh position from the C-terminus

Vpu modulation of the cellular targets CD4 and tetherin have both been proposed to involve ubiquitination. Ubiquitin, a 76 amino acid and 19 kDa moiety, may be

conjugated to proteins to modulate various functions, such as altered trafficking or to target proteins for proteasomal mediated degradation. Ubiquitin is most often conjugated to lysine residues in the target protein. The CTD of the F-MLV/GaLV chimeric Env contains two lysine residues, both of which are part of the Vpu sensitivity motif (Figure 2-3). In the light of the ability to make F-MLV Env sensitive to Vpu, each of the lysines was tested for necessity towards Vpu sensitivity. Each of the lysines was mutated to arginine, which is biophysically similar to lysine but not prone to ubiquitination. When both lysines were mutated to arginine, Vpu sensitivity was abolished (Figure 2-6D). Individually, the K18R was slightly less sensitive to Vpu, but the K11R abolished Vpu sensitivity. Hence, the lysine at the eleventh position from the C-terminus is required for Vpu sensitivity. When K18 was mutated to Q, to better resemble the F-MLV protease cleavage site (6), sensitivity was abolished (Figure 2-3). These data suggest that a positively charged amino acid is required at the eighteenth position for sensitivity toward Vpu and a lysine is specifically required at the eleventh position. Collectively, these data suggest that the Vpu sensitivity motif consists of a C-terminal alpha helix with a hydrophobic face adjacent to a positively charged face, including at least one lysine.

Loss of the processed form of a Vpu-sensitive Env in the presence of Vpu

Our lab has previously reported that Vpu specifically blocks the incorporation of the F-MLV/GaLV Env into HIV-1 particles (167). It has been reported previously that Vpu expression results in a loss of cleaved TM protein in cells (51). To ensure that the addition of Vpu does not enforce reduction in infectivity by means other than preventing

incorporation of Env into HIV-1 particles, cells were transfected with 500 ng of HIV-CMV-GFP with or without Vpu and 500 ng of different Env constructs as indicated in Figure 2-7A. Env is produced as a gp85 precursor and then processed by a cellular protease into gp70 (SU) and p15 (TM). In the case of the Vpu sensitive Envs, in the presence of Vpu, the processed or the gp70 form of the Env is lost and this correlates with the loss of Env incorporation into viral particles (Figure 2-7A). The infectivity of the pseudotyped viral particles in these experiments was also determined and Vpu sensitivity was calculated (Figure 2-7B).

To revisit the question of whether GaLV Env and CD4 contain the same Vpu target, we re-examined the sequence similarity in light of the identification of the precise Vpu sensitivity motif between the GaLV Env CTD, other gamma retroviral Env CTDs and the human CD4 CTD (Figure 2-8). Among the gammaretroviruses very closely related to GaLV, namely, KoRV and Wooly Monkey Sarcoma virus (WMSV), the amino acids contributing to Vpu sensitivity are conserved. The KoRV Env contains a K18R variation, but this CTD would be predicted to remain Vpu sensitive (Figure 2-6). All gammaretroviral Env CTDs maintained the predicted hydrophobic alpha helix, but no other Envs contained lysines at the critical 11^{th} and 18^{th} positions. In fact, the vast majority of gammaretroviral Envs contain highly conserved glutamines residues at these two positions. In contrast, the human CD4 CTD is predicted to contain an alpha helix with hydrophobic residues at the appropriate locations, and contains lysines in the positions equivalent to the 11^{th} and 18^{th} positions (Figure 2-8). Collectively, these data

suggest that GaLV Env and CD4 unexpectedly contain sequences with equivalent Vpu recognition sequences.

Discussion

In this study, the sequence in GaLV Env CTD that modulates Vpu sensitivity has been identified comprehensively to be INxxIxxVKxxVxRxK. Previous reports had identified the KRLLSEKKT sequence in CD4 to be the minimal sequence required for restriction by Vpu and was later narrowed down to EKKT in the CD4 CTD (153, 285, 308). This study, however, shows that the lysines, separated by 7 amino acids or two turns of the helix, within the KRLLSEKKT are required. The predicted motif required for restriction is essentially an alpha helix with a positively charged face that includes at least one lysine and a hydrophobic face. The alpha helix structural requirement in the CD4 CTD for Vpu-mediated downregulation has been previously reported (308).

Vpu is thought to modulate CD4 expression by inducing its polyubiquitination and subsequent degradation from the ER (298-299). The enhanced turnover and ubiquitination of CD4 in the presence of Vpu in the CD4 CTD is well documented (179, 250). Ubiquitination of proteins occurs predominantly at lysine residues. However mutational studies on the CD4 CTD have indicated a role for amino acids other than lysine in Vpu-mediated degradation (21, 174). Vpu-dependent CD4 ubiquitination is abolished only when lysine, serine, and threonine in the CD4 CTD are mutated (174). In our study to identify amino acids involved in Vpu-dependent modulation of GaLV Env,

we found that serines in the GaLV Env CTD are not necessary. The only amino acids likely to be ubiquitinated in the motif are the two lysine residues (Figures 3, 6 and 8). In fact, F-MLV Env could be made sensitive to Vpu by introduction of these two lysines in the appropriate locations of the F-MLV Env CTD (Figure 2-6).

Our finding that the membrane proximal lysine (K18) can be replaced by arginine, but not glutamine, without affecting Vpu sensitivity (Figure 2-5) appears to differ from the findings of a previous report (7). Christodoulopoulos et al. reported that mutating this lysine residue (denoted K618 in the previous report) to arginine abolished Vpu sensitivity and to glutamine partially alleviated sensitivity. The reason for this discrepancy is not known, but two key differences between this study and the previous study are notable. First, the present study analyzed the GaLV Env CTD in the context of an F-MLV Env protein; the previous study was performed with a full-length GaLV Env. Second, the output of the current study was loss of infectious particle production; the output of the previous study was loss of mature GaLV Env expression within the cell. In the present study the K18R mutation did reduce sensitivity to Vpu, but it did not abolish it. Thus, the difference between these results and previous results could simply reflect differences in sensitivity of the two assays.

In addition to identifying the motif, this current study found that when the sequences of GaLV Env CTD and CD4 CTD are aligned in the context of the Vpu sensitivity motif, the CD4 CTD also contains a sequence consistent with the GaLV Env

sensitivity motif, namely two lysines separated by two helicals of a predicted alpha helix (Figure 2-8). TASK-1, another protein reported to be targeted by Vpu (107), also contains a sequence consistent with this sensitivity motif. Tetherin is reported to be targeted by Vpu through its transmembrane region rather than its CTD; not surprising, no sequence consistent with the GaLV Env Vpu sensitivity motif is found in tetherin's cytoplasmic domain. It is likely that Vpu-based restriction of proteins by cytoplasmic tail recognition is different from transmembrane domain recognition (250)(37).

The infectivity of the F-MLV/GaLV Δ8 Env is greater than that of the full-length F-MLV/GaLV Env. These observations indicate the presence of an inhibitory/ regulatory sequence in the C-terminal eight amino acids in the GaLV Env CTD. Because these amino acids are all removed by R-peptide cleavage, their inhibitory effect presumably occurs prior to cleavage. Indeed, F-MLV/GaLV Env is not incorporated into HIV-1 particles at the same levels as wildtype F-MLV Env (Figure 2-S1). A similar phenomenon has been observed with the gammaretrovirus Env protein from the feline endogenous retrovirus RD114. This Env is poorly incorporated into lentiviral particles and the inhibitory sequence has been mapped to an acidic motif in the last six amino acids of the protein (27). The loss of incorporation is attributed to alterations in protein trafficking due to the acid motif. Altering two of the negatively charged amino acids alleviated the retroviral restriction (27). Although the C-terminal tail of GaLV Env is not as negatively charged as RD114 Env, the last eight amino acids of GaLV Env do include two negatively charged residues.

Gammaretroviral Env proteins have been important components of several gene therapy applications. Because different applications required combining different virus/glycoprotein combinations, it is important to understanding the molecular details that lead to compatibility among these combinations. This study provides an understanding of what causes incompatibility between a gammaretroviral Env and a Vpu containing lentivirus. We can now predict other glycoproteins that are likely to be incompatible with such a virus and also the information provided here could be used to eliminate this incompatibility.

In conclusion, the Vpu sensitivity motif appears to be an alpha helix on the cytoplasmic side of a membrane protein with a positively charged face including at least one lysine and a hydrophobic face. This information can potentially be used to identify additional Vpu target proteins by *in silico* screening for proteins with these properties and to develop newer lentivirus based gene transfer vectors.

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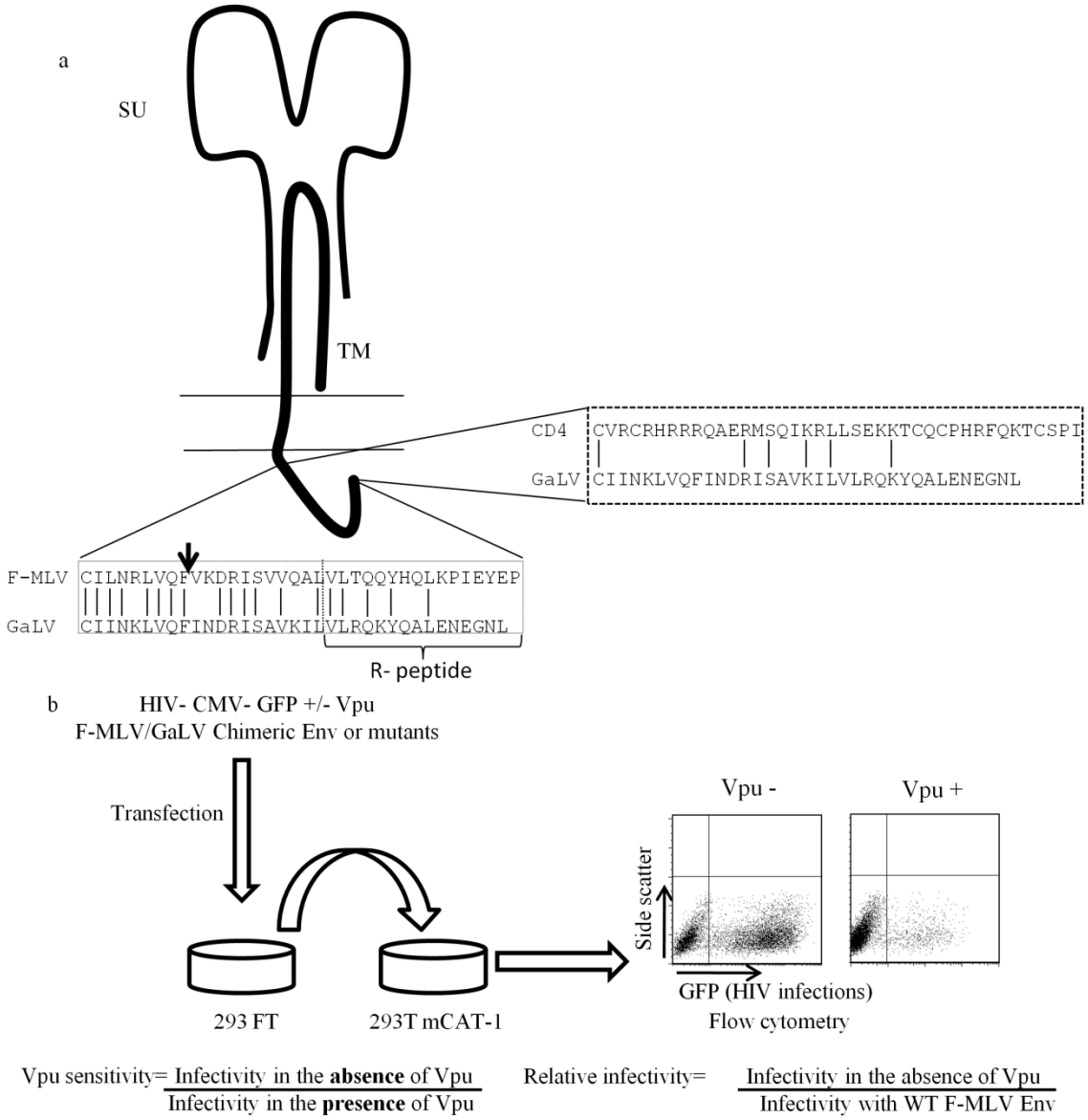
FIGURE 2-1. **Env Representation and Experimental Methodology**

(a) Schematic of the MLV and GaLV Env proteins. The amino acid sequences in the CTD of F-MLV Env, GaLV Env and CD4 are depicted. The arrow indicates the point at which the MLV Env CTD was exchanged for the equivalent sequence from GaLV Env CTD in the F-MLV/GaLV chimeric Env construct. The vertical lines indicate the alignment of the amino acid residues between the CTD of F-MLV Env and GaLV Env or between GaLV Env and CD4. (b) Schematic of the single round infectivity assay. HIV-CMV-GFP (+/- Vpu) is cotransfected with different Env constructs into 293 FT cells. Supernatant is applied to fresh 293T mCAT-1 cells and infected cells are quantitiated by flow cytometry. Projected on the plot is the side scatter (Y-axis) against the GFP fluorescence (X-axis). Vpu sensitivity is quantified as a ratio of infectivity (percent cells infected) from provirus without Vpu divided by the infectivity from a parallel assay using a provirus containing Vpu. Relative infectivity is quantified as a ratio of infectivity in the absence of Vpu to infectivity with WT F-MLV Env in the same experiment

Figure 2-1: Env Representation and Experimental Methodology

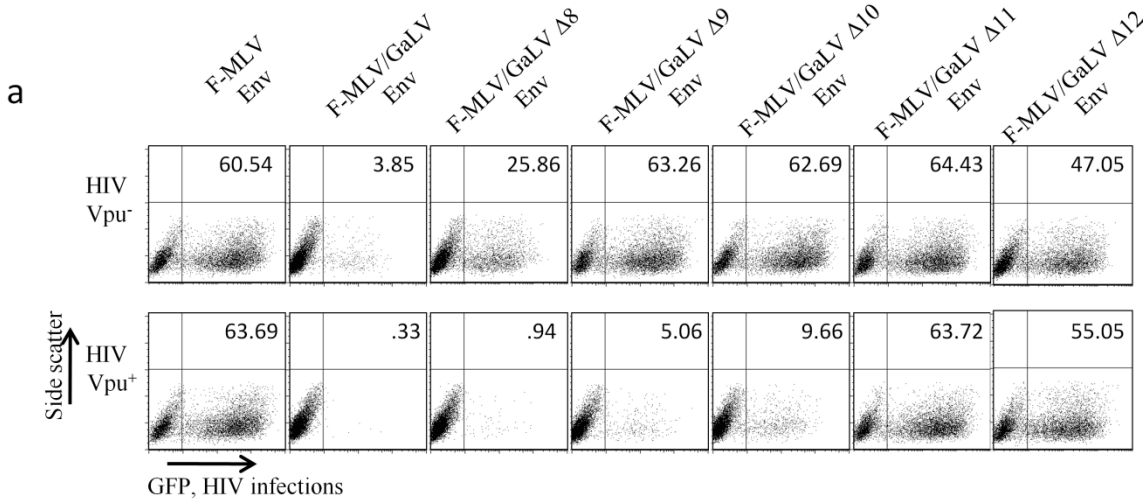


$$\text{Vpu sensitivity} = \frac{\text{Infectivity in the } \mathbf{absence} \text{ of Vpu}}{\text{Infectivity in the } \mathbf{presence} \text{ of Vpu}} \qquad \text{Relative infectivity} = \frac{\text{Infectivity in the absence of Vpu}}{\text{Infectivity with WT F-MLV Env}}$$

Figure 2-2. **HIV-1 Vpu modulates infectivity with F-MLV/GaLV Env**

(a) Infectivity plots are as described in Figure 1 and the percentage of infected cells is indicated in the dot plots. (b) Scheme of truncated mutants. 'X' represents a deleted amino acid and '~' represents an unchanged amino acid. Vpu sensitivity and relative infectivity has been calculated as in Figure 1 and is the average of two experiments. Mean relative infectivity and SD in the experiments is shown.

Figure 2-2: HIV-1 Vpu modulates infectivity with F-MLV/GaLV Env



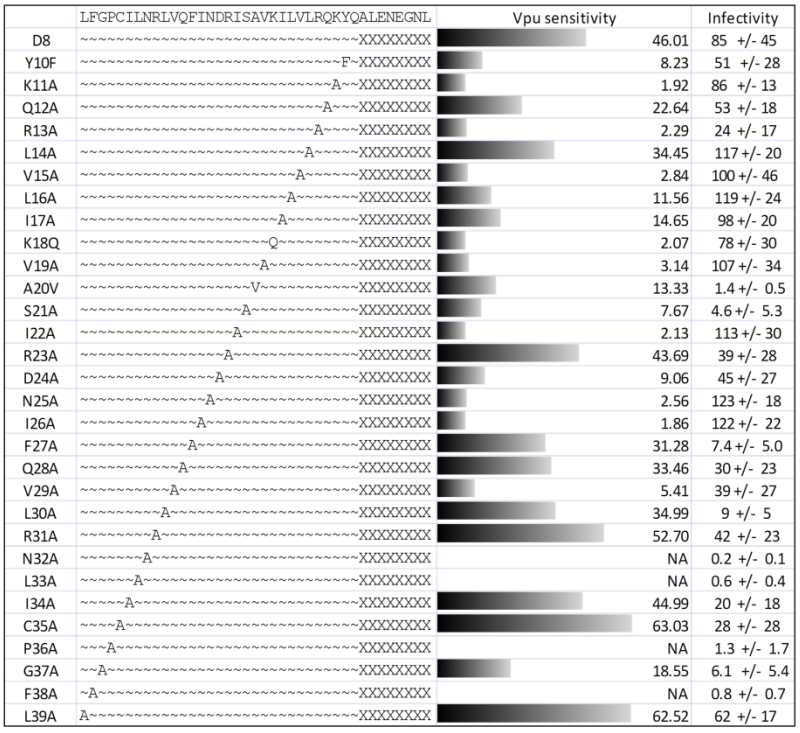
b

		Vpu sensitivity	Infectivity
F-MLV	LFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	0.6	100 +/- 0
GaLV	LFGPCILNRLVQFINDRISAVKILVLRQKYQALENEGNL	9.0	9 +/- 4
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	15.4	28 +/- 21
Δ9	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXXX	12.8	124 +/- 28
Δ10	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXXX	10.4	137 +/- 47
Δ11	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXXX	1.2	157 +/- 71
Δ12	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXXX	0.8	146 +/- 97

Figure 2-3. **Scanning mutagenesis of the GaLV CTD**

Mutants are depicted as in Figure 2. A letter at a particular position represents mutation of the amino acid in that position. Vpu sensitivity relative infectivity has been calculated as shown in Figure 1 and is the average of two experiments for mutations in amino acid residues 32 through 35; the average of three experiments for mutations of amino acids 11, 12, 13, 15, 16, 23, 27, 30, 36, 37 and 38; and the average of four experiments for mutations of residues 18, 19, 20, 22 and 26; the average of five experiments for mutations of residues 10, 17, 21, 24, 25, 28, 29, 31 and 39; and the average of seven experiments for mutation of amino acid residue 14; average of 31 experiments for Δ8 construct; NA, not applicable. Mean relative infectivity and the SD in the experiments is shown.

Figure 2-3: Scanning mutagenesis of the GaLV CTD



	LFGPCILNRLVQFINDRISAVKILVLRQKYQALENEGNL	Vpu sensitivity	Infectivity
D8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	46.01	85 +/- 45
Y10F	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~F~XXXXXXXX	8.23	51 +/- 28
K11A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~XXXXXXXX	1.92	86 +/- 13
Q12A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~~XXXXXXXX	22.64	53 +/- 18
R13A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~~~XXXXXXXX	2.29	24 +/- 17
L14A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~~~~XXXXXXXX	34.45	117 +/- 20
V15A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~~~~XXXXXXXX	2.84	100 +/- 46
L16A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~A~~~~~~XXXXXXXX	11.56	119 +/- 24
I17A	~~~~~~A~~~~~~XXXXXXXX	14.65	98 +/- 20
K18Q	~~~~~~Q~~~~~~XXXXXXXX	2.07	78 +/- 30
V19A	~~~~~~A~~~~~XXXXXXXX	3.14	107 +/- 34
A20V	~~~~~~V~~~~~XXXXXXXX	13.33	1.4 +/- 0.5
S21A	~~~~~~A~~~~XXXXXXXX	7.67	4.6 +/- 5.3
I22A	~~~~~~A~~~XXXXXXXX	2.13	113 +/- 30
R23A	~~~~~~A~~XXXXXXXX	43.69	39 +/- 28
D24A	~~~~~~A~XXXXXXXX	9.06	45 +/- 27
N25A	~~~~~~AXXXXXXXX	2.56	123 +/- 18
I26A	~~~~~~XXXXXXXX	1.86	122 +/- 22
F27A	~~~~~~XXXXXXXX	31.28	7.4 +/- 5.0
Q28A	~~~~~~XXXXXXXX	33.46	30 +/- 23
V29A	~~~~~~XXXXXXXX	5.41	39 +/- 27
L30A	~~~~~~XXXXXXXX	34.99	9 +/- 5
R31A	~~~~~~XXXXXXXX	52.70	42 +/- 23
N32A	~~~~~~XXXXXXXX	NA	0.2 +/- 0.1
L33A	~~~~~~XXXXXXXX	NA	0.6 +/- 0.4
I34A	~~~~~~XXXXXXXX	44.99	20 +/- 18
C35A	~~~~~~XXXXXXXX	63.03	28 +/- 28
P36A	~~~~~~XXXXXXXX	NA	1.3 +/- 1.7
G37A	~~~~~~XXXXXXXX	18.55	6.1 +/- 5.4
F38A	~~~~~~XXXXXXXX	NA	0.8 +/- 0.7
L39A	~~~~~~XXXXXXXX	62.52	62 +/- 17

Vpu sensitivity seqeunce
INxx**I**xx**VK**xx**V**x**R**x**K**

Figure 2-4. **RSV/GaLV Env is Vpu sensitive**

CTD Amino acid sequences of the indicated Envs are depicted. Vpu sensitivity with F-MLV Env, Δ8 and Δ12 Envs are the average of 4 experiments, the average of 3 experiments for RSV/GaLV Env and the average of 2 experiments for RSV Env. Mean relative Infectivity +/- SD in the experiments is shown .

Figure 2-4: RSV/GaLV Env is Vpu sensitive

		Vpu sensitivity	Infectivity
F-MLV	CILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	1.35	100 +/- 0
RSV	CLLQIVCGNIRKMINNSISYHTEYKKLQKACGQPESRIV	1.02	59 +/-24
RSV/GaLV	CILNRLVQFINDRISAVKILVLRQKYQALENEGNL	21.11	6.7 +/-7.9
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	53.86	57 +/-24
Δ12	~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXXXXXX	1.12	96 +/-8

Figure 2-5. **Vpu sensitivity requires a predicted alpha helix**

(a) Amino acid sequences are indicated as in Figure 2. Vpu sensitivity and relative infectivity is calculated as shown in Figure 1 and is the average of three experiments for mutations of residues 11, 14 and 17; the average of five experiments for mutation of residue 23 and the average of eight experiments for mutation of residues 20 and 26; the average of 11 experiments for F-MLV and Δ8 Envs. Mean relative infectivity with SD in the experiments is shown. (b) Helix representation of GaLV Env CTD is depicted starting from L33 at position d and ending at Y10 at position f. Amino acids required for Vpu sensitivity are underlined.

Figure 2-5: Vpu sensitivity requires a predicted alpha helix

a

		Vpu sensitivity	Infectivity
F-MLV	LFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	1.01	100 +/- 0
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	35.59	38 +/- 26
K11P	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~P~~XXXXXXXX	1.23	141 +/- 35
L14P	~~~~~~~~~~~~~~~~~~~~~~~~~~~P~~~~~XXXXXXXX	0.75	133 +/- 65
I17P	~~~~~~~~~~~~~~~~~~~~~~~~P~~~~~~~~XXXXXXXX	0.76	120 +/- 24
A20P	~~~~~~~~~~~~~~~~~~~~P~~~~~~~~~~XXXXXXXX	1.69	91 +/- 10
R23P	~~~~~~~~~~~~~~~~~~P~~~~~~~~~~~~XXXXXXXX	0.97	125 +/- 58
I26P	~~~~~~~~~~~~~~~P~~~~~~~~~~~~~~~~~XXXXXXXX	1.87	76 +/- 20

b

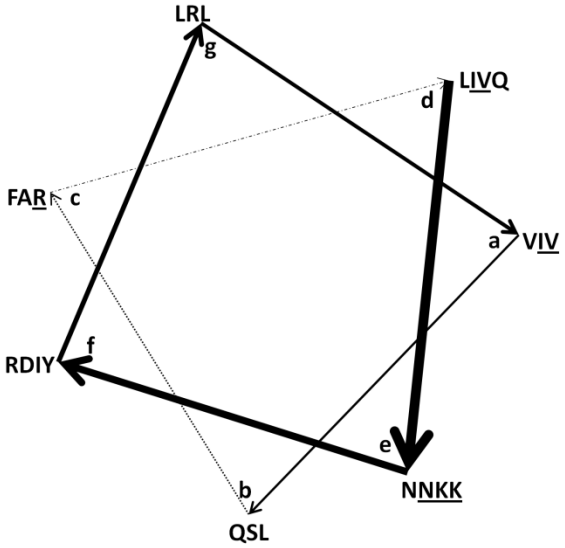


Figure 2-6. **F-MLV Env can be made sensitive to Vpu**

(a) F-MLV and GaLV Env CTD along with the Vpu sensitivity motif are aligned and the differences between F-MLV and GaLV Env CTD within the motif are boxed. (b) The alpha helical representation is of the F-MLV Env CTD starting from L34 at position d and ending at Y11 at position f. F-MLV Env CTD differs from GaLV Env CTD within the Vpu sensitivity motif at the underlined positions (c) F-MLV Env CTD has been mutated to more closely resemble the Vpu sensitivity motif. Amino acid sequences are indicated as in Figure 2-2. Vpu sensitivity and relative infectivity are calculated as shown in Figure 2-1 and is the average of 6 experiments for F-MLV Env mutants and the average of 9 experiments for the WT F-MLV Env. (d) The lysines that can render F-MLV Env sensitive to Vpu have been mutated in the GaLV Env CTD. Data for K18Q Env from Figure 2-3 has been included for comparison. Vpu sensitivity shown is the average of 4 experiments for the Δ8 and F-MLV/GaLV K11R, K18R Envs; the average of 3 experiments for K11R and K18R single mutation Envs. Mean relative infectivity and SD in the experiments are shown.

Figure 2-6: F-MLV Env can be made sensitive to Vpu

a

```
F-MLV      CILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP
GaLV       CIINKLVQFINDRISAVKILVLRQKYQALENEGNL
Motif      ---------IN--I--VK--V-R-K-----------
```

b

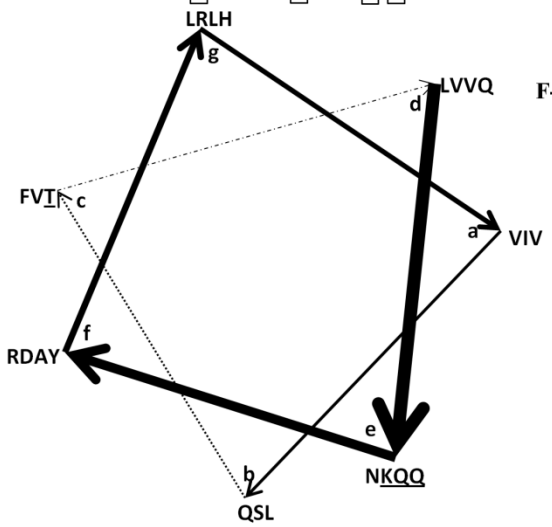


Figure 6

c **F-MLV Env CTD**

		Vpu sensitivity	Infectivity
F-MLV	LFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	1.00	100 +/- 0
Q12K	~~~~~~~~~~~~~~~~~~~~~~~~~~~~K~~~~~~~~~~	2.75	57 +/- 25
Q19K	~~~~~~~~~~~~~~~~~~~~K~~~~~~~~~~~~~~~~~	0.94	90 +/- 31
Q12K,Q19 K	~~~~~~~~~~~~~~~~~~~~K~~~~~~K~~~~~~~~~~	17.60	64 +/- 22
K26N,Q12K	~~~~~~~~~~~~~~~N~~~~~~~~~~~~~K~~~~~~~~~~	1.14	14 +/- 10
K26N,Q12K,Q19K	~~~~~~~~~~~~~~~N~~~~~~K~~~~~~K~~~~~~~~~~	8.92	105 +/- 29

d **GaLV Env CTD**

F-MLV/GaLV	LFGPCILNRLVQFINDRISAVKILVLRQKYQALENEGNL	Vpu sensitivity	Infectivity
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	76.3	82 +/- 26
K11R,K18R	~~~~~~~~~~~~~~~~~~~~R~~~~~~R~~XXXXXXXX	1.22	86 +/- 29
K11R	~~~~~~~~~~~~~~~~~~~~~~~~~~R~~XXXXXXXX	1.66	18 +/- 7
K18R	~~~~~~~~~~~~~~~~~~~~R~~~~~~~~~XXXXXXXX	15.72	32 +/- 33
K18Q	~~~~~~~~~~~~~~~~~~~~Q~~~~~~~~~XXXXXXXX	2.06	78 +/- 30

Figure 2-7. **Loss of the processed form of a Vpu sensitive Env in the presence of Vpu**

(a) 293 FT cells were transfected with HIV-CMV-GFP +/- Vpu and different Envs as indicated. Western blot analysis was performed on the transfected cells and pelleted viral supernatants. Representative image of the experiments performed has been shown. (b) Vpu sensitivity of the various envelopes used in these experiments is shown and is an average of 19 independent experiments. Error bars represent standard deviations from the mean from 19 independent experiments.

Figure 2-7: Loss of the processed form of a Vpu sensitive Env in the presence of Vpu

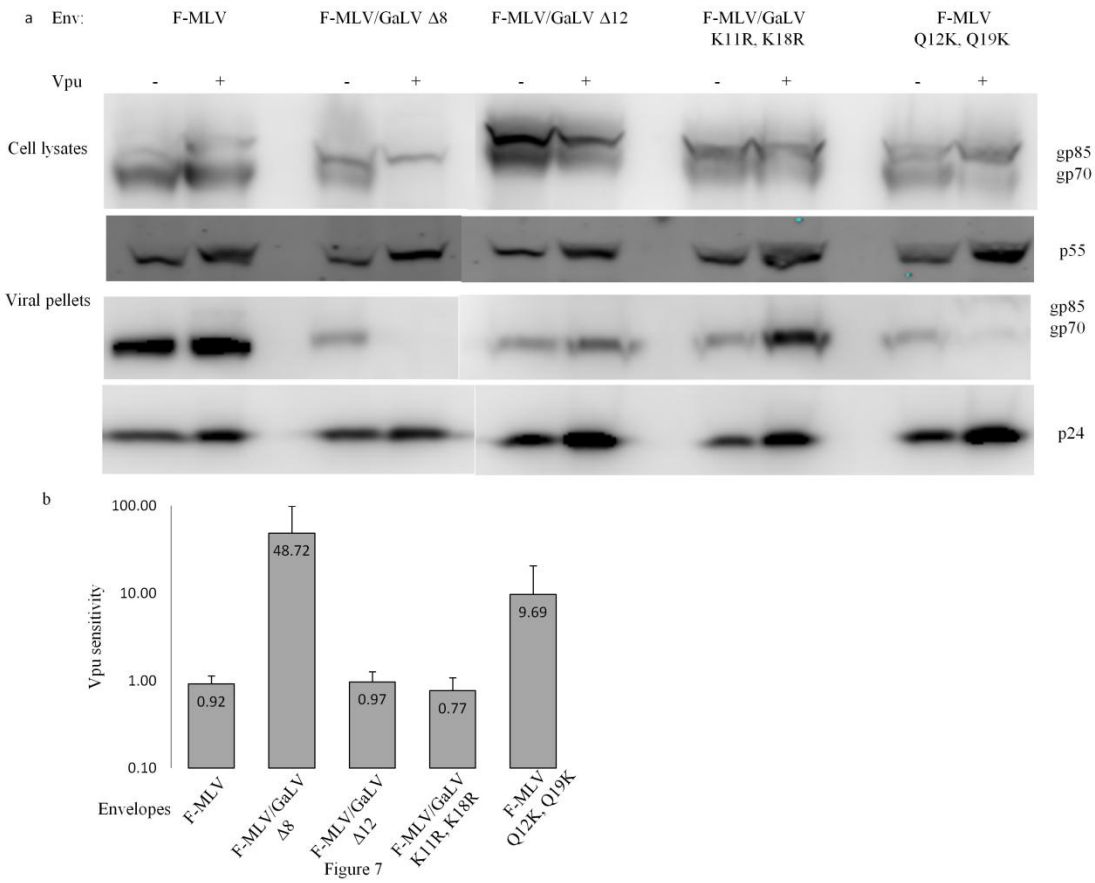


Figure 7

Figure 2-8. **CTD sequences of Gammaretroviruses**

Alignment the CTDs from GaLV Env, WMSV Env, KORV Env, Moloney murine leukemia virus (MoMLV) Env, F-MLV Env, Feline leukemia virus (FLV) Env, Kirsten murine leukemia virus (KMVL Env), xenotropic murine leukemia related virus (XMRV), endogenous retroviruses (RV) and human CD4 CTD along with the Vpu sensitivity motif. Dark boxes highlight the critical lysine positions, light boxes highlight the other residues critical for Vpu sensitivity in GaLV Env.

Figure 2-8: CTD sequences of Gammaretroviruses

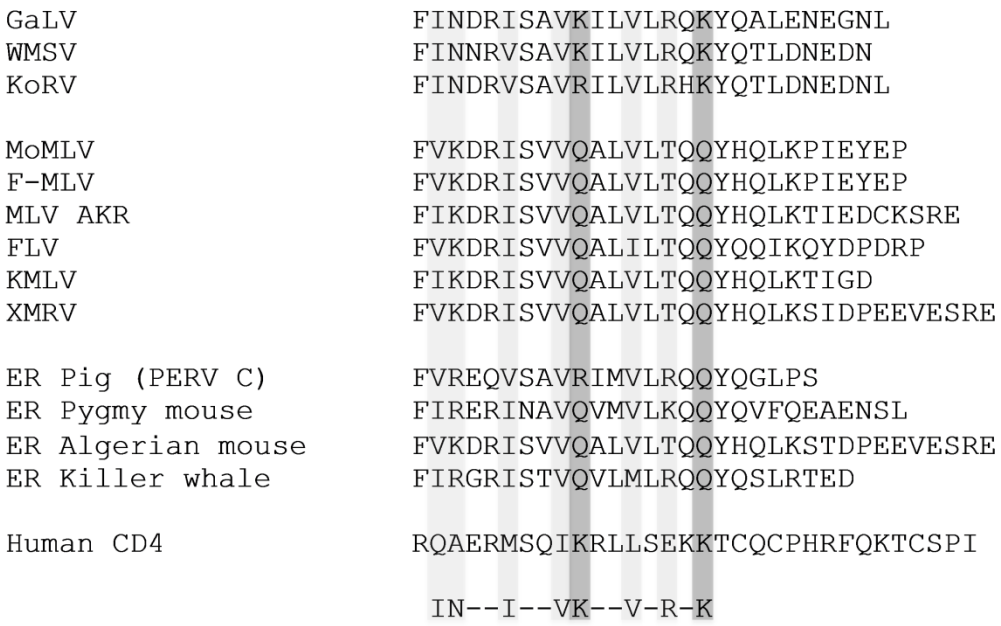


Figure 2-S1. **Incorporation of Env into viral particles**

(a) A representative Western blot showing Env and HIV-1 Gag expression in transfected cells and in viral pellets. (b) Bar graph shows the infectivity or pseudotyped HIV virions with each of the Envelopes indicated. Infectivity shown here is the average of 4 experiments and the whiskers represent the standard deviation.

Figure 2-S1: Incorporation of Env into viral particles

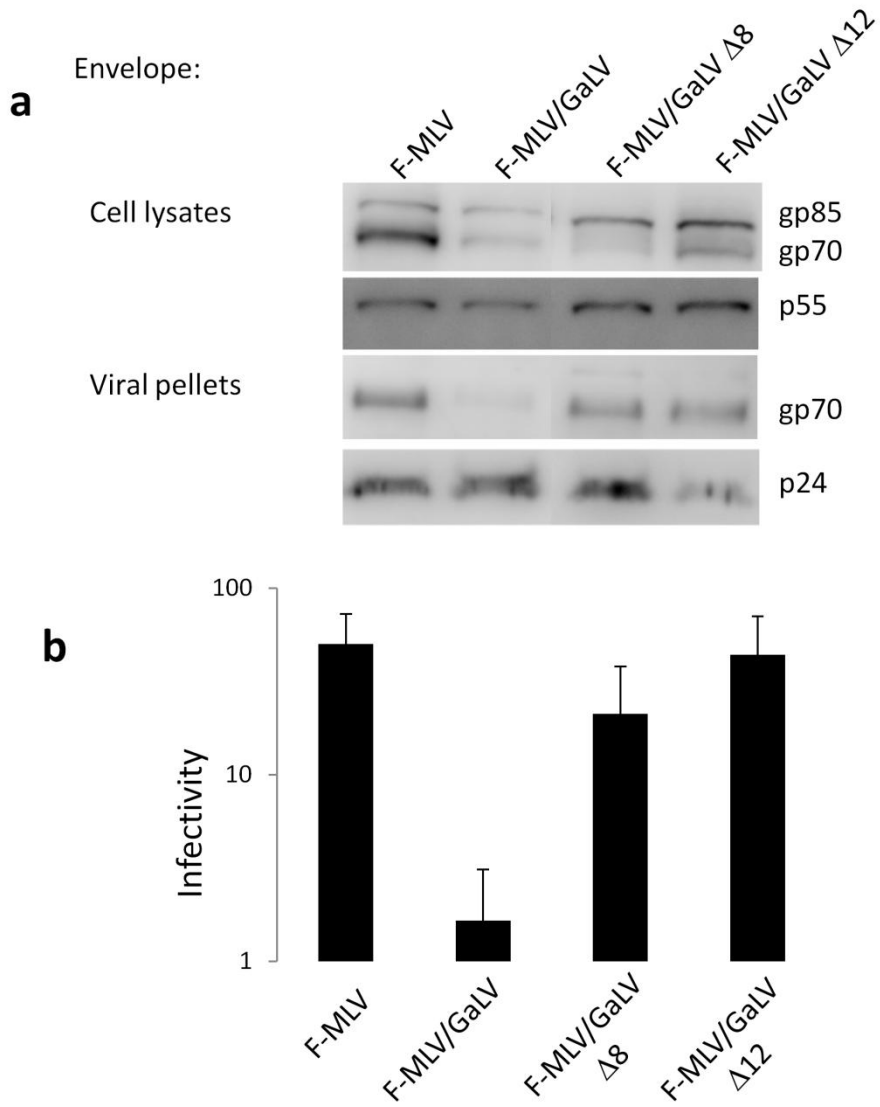


Figure 2-S2. **Alanine scanning mutagenesis**

Amino acid sequences are depicted as in Figure 2-2. Vpu sensitivity has been calculated as in Figure 2-1 and is the average of two experiments for the 5 alanine mutants and the average of three experiments for the F-MLV and Δ8 mutants. Mean relative infectivity and SD in the experiments are shown.

Figure 2-S2: Alanine scanning mutagenesis

	LFGPCILNRLVQFINDRISAVKILVLRQKYQALENEGNL	Vpu sensitivity	Infectivity
F-MLV	LFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	0.59	100 +/- 0
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	49.42	104 +/- 85
9-13A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AAAAAXXXXXXXX	1.04	131 +/- 18
14-18A	~~~~~~~~~~~~~~~~~~~~~~~~~AAAAA~~~~~XXXXXXXX	1.29	47 +/ 41
19-23A	~~~~~~~~~~~~~~~~~AAAAA~~~~~~~~~~XXXXXXXX	NA	0.2 +/- 0
24-28A	~~~~~~~~~~~AAAAA~~~~~~~~~~~~~~~~XXXXXXXX	1.31	8.8 +/- 9.7

Figure 2-S3. **Alanine scanning mutagenesis**

Amino acid sequences are depicted as in Figure 2-2. Vpu sensitivity has been calculated as in Figure 2-1 and is the average of 3 experiments for the mutants 19A-20V, 21-22A, 25-26A, 27-28A, the average of 4 experiments for 9-10A, 11-12A, 15-16A, 23-24A, the average of 5 experiments for 13-14A, the average of 6 experiments for 17-18A and the average of 8 experiments for the F-MLV and D8 mutants. Mean Relative percentage Infectivity +/- SD of HIV-1 pseudotyped with the mutant Envs in the absence of Vpu is shown

Figure 2-S3: Alanine scanning mutagenesis

	LFGPCILNRLVQFINDRISAVKILVLRQKYQALENEGNL	Vpu sensitivity	Infectivity
F-MLV	LFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP	0.95	100 +/- 0
Δ8	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XXXXXXXX	49.57	65 +/- 33
9-10A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AAXXXXXXXX	28.82	32 +/- 16
11-12A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~XXXXXXXX	1.11	45 +/- 26
13-14A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~XXXXXXXX	2.17	46 +/- 27
15-16A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~XXXXXXXX	1.77	124 +/- 83
17-18A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~~~XXXXXXXX	1.35	48 +/- 50
19A,20V	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~VA~~~~~~~~~~XXXXXXXX	1.26	108 +/- 33
21-22A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~~~~~XXXXXXXX	3.69	94 +/- 42
23-24A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~~~~~XXXXXXXX	NA	0.4 +/- 0.2
25-26A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~~~~~XXXXXXXX	1.06	118 +/- 67
27-28A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~~~~~~~XXXXXXXX	NA	1.1 +/- 0.7

III. Functional complementation of a model target to study Vpu sensitivity

Sanath Kumar Janaka, Jared Faurot and Marc C Johnson

This study has been submitted to PLoS One and is under review (113)

Abstract

The cytoplasmic tail domain (CTD) of Gibbon Ape Leukemia virus (GaLV) Envelope (Env) is modulated by the HIV-1 accessory protein Vpu. Using Env functional complementation, we sought to determine if Vpu sensitivity requires that all of the CTDs in the Env trimer be from GaLV Env. In some cases, the mixed trimers with Vpu sensitive and insensitive CTDs remained Vpu sensitive. However, deletion of the CTD from any of the three subunits abolished Vpu sensitivity.

Unlike most gammaretrovirus Env proteins, GaLV Env cannot pseudotype HIV-1 particles (50, 269). This incompatibility was found to be dictated by the CTD of GaLV Env (51, 115, 167). Recently, we and others have reported that this incompatibility is primarily caused by the HIV-1 accessory protein Vpu, which prevents Env proteins with the GaLV Env CTD from being incorporated into viral particles (51, 115, 167). The CTDs of gammaretroviral Env proteins are predicted to be alpha helical and likely form coiled-coil trimers (78, 165, 276). An alanine scanning mutagenetic analysis of the GaLV Env CTD helped identify the sequence conferring Vpu sensitivity on GaLV Env CTD to be INxxIxxVKxxVxRxK (115). The motif contains two lysine residues that are also found in the corresponding positions of the CD4 CTD, another target of Vpu (115). We observed that mutation of the hydrophobic residues believed to form the interface of the CTD coiled-coil alleviated Vpu sensitivity. We therefore wanted to understand if this putative coiled-coil is required for the Env trimer to be Vpu sensitive, and whether all three helices in this trimer must contain the sensitivity motif. To answer these questions, we chose to take advantage of functional complementation of receptor-binding defective or fusion defective MLV Envs with the CTDs containing a Vpu-sensitive or a Vpu-resistant helix (313).

The Friend murine leukemia virus (F-MLV) Env protein is a trimer (82), and the individual monomers are made up of a 70 kDa surface subunit (SU) and a 15 kDa transmembrane unit (TM)(18, 20). SU provides the receptor binding function (15-16, 98, 172, 209) and TM provides the fusion function (43, 120, 314). Mutations conferring receptor binding defects and fusion defects have been identified, and some pairs of

defects on two separate Env molecules can functionally complement each other (313-314). Functionally defective Env constructs lacking receptor binding function or fusion function were created, as described previously (313). The D84K mutation, conferring receptor binding defect (BD) on the Env and the L493V mutation, conferring a fusion defect (FD) on the Env are able to functionally complement one another (313). Env constructs with these mutations were generated in the context F-MLV with a GaLV Env Δ8 CTD (Vpu sensitive helix) or an F-MLV Env CTD (Vpu resistant helix) (Figure 3-1). GaLV Env Δ8 CTD lacks the C-terminal eight amino acids from GaLV Env and was used because the last eight amino acids of GaLV Env reduces infectivity with HIV-1 cores irrespective of Vpu (115). Also, BD and FD Envs with most of the CTD deleted (Δ25) were generated.

Gammaretroviral Envs with different CTDs complement each other

Infectivity assays with HIV-CMV-GFP were performed with pairs of BD and FD Env constructs to confirm that the proteins are able to complement each other as previously described (313). The individual BD and FD Envs do not produce infectious particles on their own (Figure 3-2A) but each BD construct was able to complement each FD Env with variable efficiency (Figure 3-2B). Infectivity with wildtype (WT) F-MLV Env in each experiment was used to normalize the data and obtain complementation efficiency. While highly related Envs have been known to form mixed trimers (41), infectivity data from this assay shows that Envs with different CTDs can still complement each other functionally.

Mixed Env trimers can be Vpu sensitive

To understand the Vpu sensitivity of the different Env trimers, pairwise transfections with complementation pairs were performed in the presence or absence of Vpu as previously described ((115), Figure 3-2B). Vpu sensitivity was calculated as the ratio of infectivity in the absence of Vpu to the infectivity in the presence of Vpu (Figure 3-3). As expected, the Env trimers that contained only F-MLV CTDs was Vpu resistant and the Env trimers that contained only GaLV Δ8 CTDs was Vpu sensitive (Figure 3-2B, 3). With mixed trimers, infectivity of the complementation pair of a BD F-MLV/GaLV Δ8 (Vpu sensitive) with a FD F-MLV Env (Vpu resistant) was 20-fold higher in the absence of Vpu (Figure 3-2B, 3), suggesting that Vpu sensitivity does not require all three CTDs to contain the sensitivity sequence. However, infectivity of the reciprocal complementation pair, with a FD F-MLV/GaLV Δ8 Env and a BD F-MLV Env, was only 1.5 fold higher in the absence of Vpu (Figure 3-2B, 3). This suggests that the SU domain of Env may be able to partially complement independent of its Vpu-sensitive TM subunit. Modularity of the receptor binding function has been previously reported (13).

Vpu sensitivity is conferred on a functionally complemented Env complex only if all the three CTDs are present

The hydrophobic amino acids in the 'vpu-sensitivity sequence' may form a coiled-coil interface in the predicted alpha helix (276), and so we tested whether F-MLV/GaLV Env proteins with fewer than three CTDs could remain Vpu sensitive. For this, we complemented F-MLV Env Δ25 with F-MLV and F-MLV/GaLVΔ8 Env. F-

MLV Δ25 remains functional and Vpu resistant (115, 167). Despite the lack of CTD, functional complementation occurred with each of these pairs, but the pairs were all Vpu resistant (Figure 3-2B and 3). Loving et al. (165) have shown with electron cryotomography reconstructions that the TM of a full length Env is in the form of a closely packed density perpendicular to the membrane prior to R-peptide cleavage. They suggest that TM is held in this closely packed form by CTD-CTD interactions that are relieved by R-peptide cleavage. Together, these pieces of data support a model wherein the gammaretroviral Env CTD forms a trimeric helix bundle and that Vpu sensitivity can only be conferred in the context of this structure.

Acknowldegements

HIV-CMV-GFP was kindly provided by Dr. Vineet Kewalramani. 293T-mCAT-1 cell line was a kind gift from Dr. Walter Mothes. This study was funded by U.S. Public Health Service grants R21 AI087448 and R01 AI73098. We would like to thank Dr. Devon Gregory for helpful discussions. We would like to thank other lab members for providing technical assistance during the length of the study.

Figure 3-1 **Schematic of the gammaretroviral Env proteins**

The amino acid sequences in the CTD of F-MLV Env and GaLV Env are depicted. Truncations in the CTD of the Envs, F-MLV Δ25 and F-MLV/GaLV Δ8 are indicated. The cartoon has been adapted from Figure 1 of (115).

Figure 3-1: Schematic of the gammaretroviral Env proteins

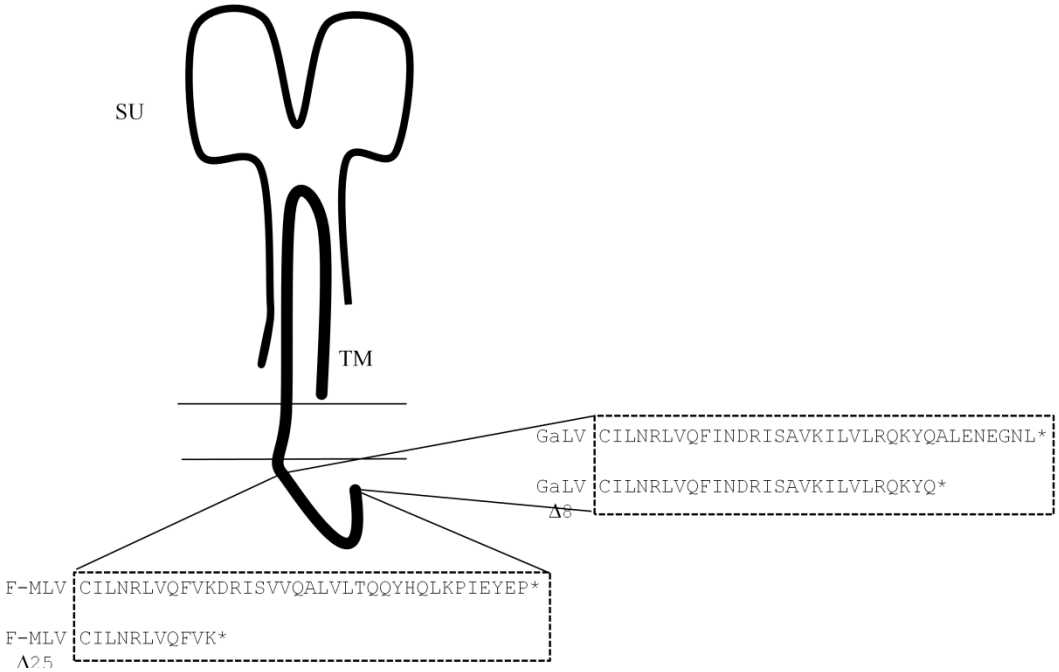


Figure 3-2 **Defective Env pairs with different CTDs complement each other functionally**

(A) Infectivity of the indicated Envs relative to infectivity with F-MLV Env. Data shown is the average of two experiment s. (B)Infectivity of complementation pairs in the presence or absence of Vpu, relative to infectivity with F-MLV Env. Data shown is the average of three experiments and the error bars indicate SD in the experiments. Welch's T- test was performed to determine p- values, indicative of statistical significance. NS- Non significant; BD- Binding defective; FD- Fusion defective.

Figure 3-2: Defective Env pairs with different CTDs complement each other functionally

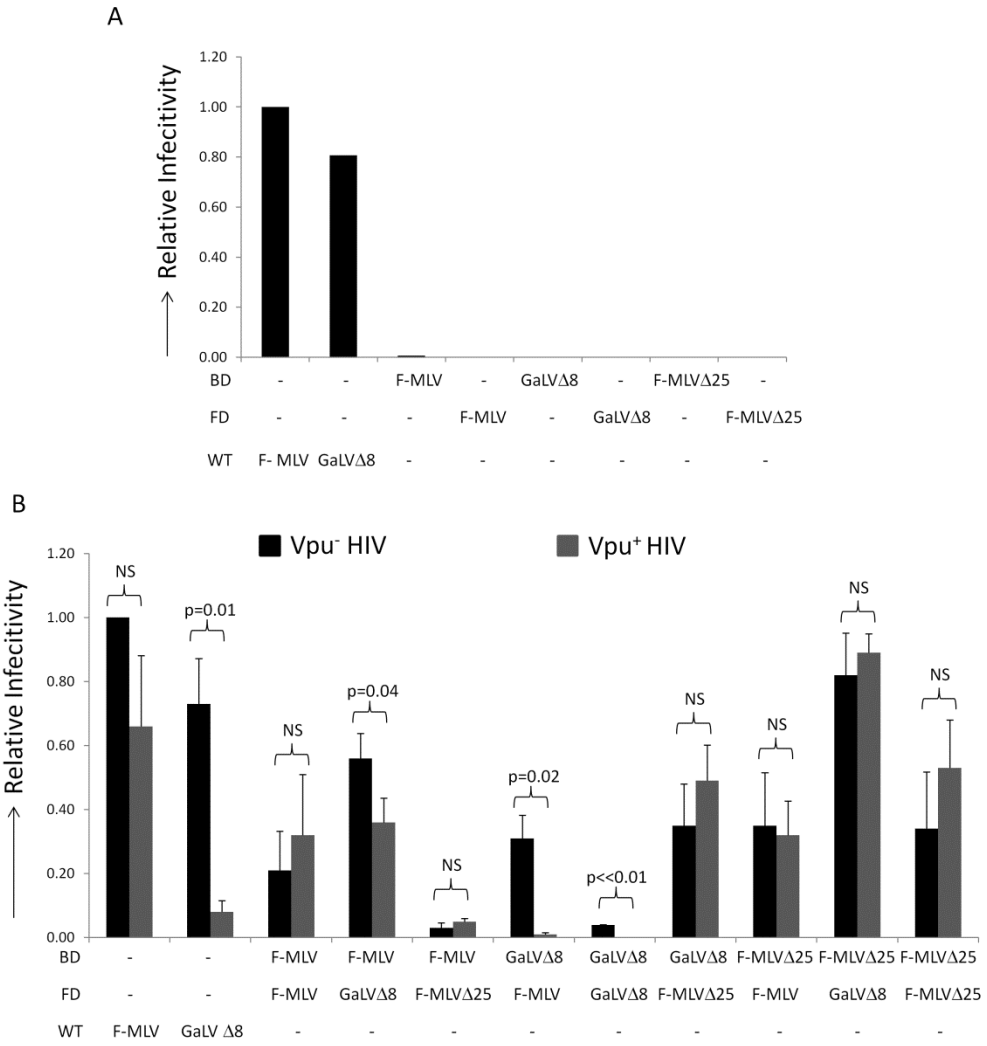
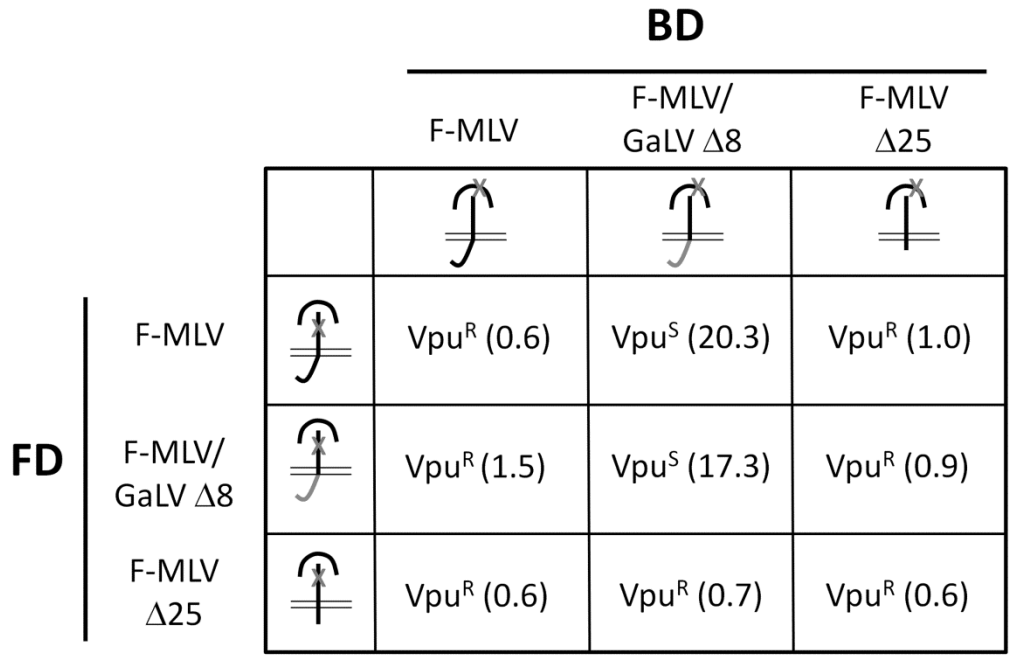


Figure 3-3: **Mixed Env trimers can be Vpu sensitive**

Sensitivity to Vpu of each complementation pair is indicated. The degree of Vpu sensitivity is indicated within parantheses as a ratio of infectivity in the absence of Vpu to infectivity in the presence of Vpu. Data shown is the average of three independent experiments in Figure 3-2B.Vpu^{R}- Vpu resistant; Vpu^{S}- Vpu sensitive.

Figure 3-3: Mixed Env trimers can be Vpu sensitive



IV. Vpu appropriates overlapping features for downmodulation of distinct targets, tetherin and gibbon ape leukemia virus envelope

Tiffany M. Lucas,[1] Sanath Kumar Janaka,[1] Edward B. Stephens, and Marc C. Johnson

1 – These authors contributed equally to this manuscript

Note: The research presented in chapter IV was conducted equally between T.M. Lucas and S.K. Janaka. The manuscript was written by T.M. Lucas and revised by all authors.

This work has been published in PLoS One (166).

Abstract

During human immunodeficiency virus 1 (HIV-1) assembly the host proteins CD4, the HIV-1 receptor, and tetherin, an interferon stimulated anti-viral protein, reduce viral fitness. To counteract these host proteins, the HIV-1 accessory gene Vpu is thought to target both CD4 and tetherin through two distinct mechanisms. Modulation of CD4 likely occurs through proteasomal degradation from the endoplasmic reticulum. The exact mechanism of tetherin modulation is less clear, with possible roles for degradation and alteration of protein transport from and to the plasma membrane. Investigation of Vpu function historically relied on different assays for CD4 and tetherin, many of which

require exogenously expressed Vpu, potentially leading to variability in expression. Thus, few studies have investigated these two Vpu functions in parallel assays, making it difficult to draw direct comparisons between Vpu targeting of tetherin and CD4.

Here, we present a rapid assay to simultaneously investigate Vpu-targeting of both tetherin and a CD4-like stand-in, gibbon ape leukemia virus envelope (GaLV Env) with a comprehensive mutagenic scan of Vpu in its native proviral context to identify features required for activity. We previously reported that Vpu modulates the envelope protein from GaLV and prevents its incorporation into HIV-1 particles, presumably in a CD4-like manner through recognition of a shared sensitivity motif in the cytoplasmic tails of CD4 and GaLV Env. Interestingly, we observed considerable overlap in the Vpu sequences required to modulate tetherin and GaLV Env. We found that features in the cytoplasmic tail of Vpu, specifically within the cytoplasmic tail hinge region, were required for modulation of both tetherin and GaLV Env. We observed a role for previously reported critical transmembrane amino acids in the restriction of tetherin but not GaLV Env. We propose that Vpu may target both proteins in a mechanistically similar manner, albeit in different cellular locations.

Introduction

Vpu is an 81-86 amino acid, type-1 transmembrane protein found in HIV-1 and a few closely related strains of SIV. Vpu modulates a wide range of targets including the host proteins CD4, tetherin, IkB, MHC-II, NTB-A, and the gammaretroviral gibbon ape leukemia virus (GaLV) envelope (Env) (28-29, 52, 110, 116, 169, 199, 259, 300). Of these functions, Vpu's abilities to degrade cellular CD4 and tetherin (BST-2, CD137) have been the best described (30, 92, 203, 243). CD4 is the primary receptor for HIV-1. Vpu targets newly synthesized CD4 in the rough endoplasmic reticulum (RER) through interactions between the cytoplasmic tails (CT) of Vpu and CD4, recruiting the Skp1-Cullin-β-TrCP E3-ubiquitin ligase complex, resulting in the subsequent proteasomal degradation of CD4 (29, 48, 85, 175, 178, 251, 297). The cytoplasmic tail (CT) of Vpu is unambiguously required for CD4 modulation, but it is disputed whether the membrane spanning domain (MSD) also plays a specific role (103, 173, 244, 252, 279).

Tetherin is an interferon inducible, type-II transmembrane anti-viral protein with a C-terminal GPI-anchor. Tetherin, as its name suggests, "tethers" many budding enveloped viruses or viral like particles to the plasma membrane (PM), including retroviruses, Ebola, Kaposi sarcoma-associated herpes virus (KSHV) and influenza virus like particles (199-200, 215, 291). Vpu-mediated antagonism of tetherin requires an interaction between the MSDs of Vpu and tetherin, but as of yet, there is no consensus on the precise mechanism by which Vpu modulates tetherin activity. Vpu has been reported to reduce tetherin surface expression by altering the rate of recycled and/or restricting

newly synthesized tetherin from reaching the PM (72, 74, 97, 187, 249, 263). However, it has also been reported that Vpu can modulate tetherin activity in the absence of surface downmodulation and intracellular depletion (189). Some studies suggest that tetherin can be degraded through β-TrCP mediated targeting to lysosomes or the proteasome (68, 176, 187).

Although the mechanisms for CD4 and tetherin antagonism are believed to be distinct, evidence suggests that Vpu contains some shared features required for modulation of both proteins. For instance, complete proscription of either target requires two critical serines housed in the Vpu cytoplasmic tail, which is also required for interaction with β-TrCP and degradation of tetherin or CD4 (176, 178, 254). Vpu mutants lacking these serine residues retain some activity against tetherin but not CD4 (22, 74). Direct parallels between Vpu modulation of tetherin and CD4 are difficult to draw due to differences in the assays employed. Studies investigating tetherin antagonism have relied heavily on detection of viral particle release, through protein release or infectious virus production, although some studies have also measured tetherin modulation directly. Reports on CD4 down-modulation typically rely on biochemical assays measuring total protein or surface expression. Additionally, Vpu studies have used different cell types, multiple methods of introducing CD4 or tetherin targets (endogenous or exogenous), and different methods of producing Vpu (e.g., native or codon-optimized, contained in the provirus or introduced *in trans*). Employment of these disparate protocols limits the ability to directly compare different studies.

We and others found that Vpu prevents GaLV Env incorporation into HIV-1 particles, likely through a shared structural recognition motif in the Env cytoplasmic tail that resembles the critical motif found in the cytoplasmic tail of CD4 (52, 116, 169). This sensitivity motif is conserved and is transferrable to confer sensitivity in previously insensitive proteins (116). Based on these findings, we currently believe Vpu recognizes GaLV Env as a CD4 analogue. Like CD4, GaLV Env is packaged into the virus in the absence of Vpu, however, unlike CD4, GaLV Env can form infectious pseudotyped virus to assess incorporation of the target protein. We therefore used GaLV Env as a surrogate for CD4 in infectivity. Modulation of GaLV Env by Vpu is sensitive and well suited for a comparative study with the modulation of tetherin by Vpu. By employing GaLV Env, constraint of both targets can be studied in the same cell type using Vpu encoded in the provirus with infectivity as the output for both.

Materials and Methods

Cell lines. The human endothelial kidney (HEK) 293FT, 293 mCAT-1, and 293 TVA (155) cells were obtained from Invitrogen, W. Mothes, and J. Young, respectively. All cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418.

Plasmids. The NL4-3 derived HIV-CMV-GFP was kindly provided by Vineet KewalRamani. This clade B provirus lacks Env, Vpr, Vpu, Nef, and Vif, and contains a

green fluorescent protein (GFP) gene in place of the Nef gene under the control of a cytomegalovirus (CMV) promoter. The gene expressing the far red fluorescent protein E2Crimson was engineered into this construct to exactly replace the GFP gene to produce HIV-CMV-E2Crimson. A library of HXB2 Vpu mutants was derived from a previously described HXB2 parent Vpu (103). Mutations to the Vpu gene were generated either by linker insertion, or by two-step PCR, and the genes were subsequently subcloned into the unique NheI and AscI sites, which were engineered immediately upstream and downstream of the natural location of the Vpu gene in HIV-CMV-E2Crimson. The human tetherin expression construct tetherin-HA (219) was kindly provided by P. Bieniasz. The VSV-G, MLV/GaLV Env, and RSV ΔCT expression constructs have been described previously (131, 169).

Infectivity analysis. 293FT cells were plated in 6-well plates and allowed to reach 60% confluency prior to transfection. For tetherin studies, 293FT cells were transfected with the following expression constructs: provirus (425 ng) and VSV-G (25 ng) with or without 12.5 ng of tetherin-HA in a total of 500 ng. For GaLV Env assays, cells received 500 ng provirus, 25 ng of RSV Env ΔCT, and 475 ng MLV/GaLV Env (GaLV Env) (116, 169). Plasmids were transfected with polyethylenimine (PEI) at a concentration of 1ug DNA per 4ul (1 mg/1 ml stock concentration) and media was replaced 6 hours later. At 48h post-transfection, media containing virus was collected and frozen overnight. For tetherin studies, the media was used to transduce 293T mCAT-1 cells expressing the murine leukemia virus Env receptor (mCAT-1). For GaLV Env studies, media was used in parallel to transduce both 293 mCAT-1 cells and 293T TVA, which expresses the

Rous sarcoma virus receptor (TVA). Two days later, cells were collected, fixed with 4% paraformaldehyde and analyzed by flow cytometry using an Accuri flow cytometer. Cells transduced by virus were gated by E2Crimson expression in the FL4 channel to determine viral infectivity.

Results

To determine specific regions of Vpu mediating antagonism of tetherin and GaLV Env, we generated a library of HXB2 Vpu mutants and introduced them into a reduced HIV-1 clade B proviral construct containing an E2Crimson reporter gene (Figure 4-1A). HXB2 Vpu was used, as several mutants had previously been generated (103). For tetherin modulation assays, each provirus was transfected with a VSV-G expression plasmid alone, or in combination with an HA-tagged tetherin expression construct, kindly provided by P. Bieniasz (219) (Figure 4-1B, left). Vpu activity was measured by comparing infectivity in the presence and absence of tetherin. For assaying GaLV Env modulation, an internally controlled system was used where each mutant was transfected with a mixture of plasmids expressing the previously described Vpu-sensitive chimeric MLV Env containing the GaLV Env cytoplasmic tail, herein referred to simply as GaLV Env, and a Vpu-insensitive Rous sarcoma virus Env lacking the cytoplasmic tail (RSVΔCT) (Figure 4-1B, right). Virus was collected and used to transduce 293T mCAT-1, expressing the MLV Env receptor (mCAT-1) and 293T TVA, which expresses the RSV receptor (TVA). Vpu activity was measured by comparing the ratio of RSV Env pseudotyped infectious virus to MLV Env pseudotyped infectious virus. For both assays,

infections were quantified by flow cytometry, and activity was expressed by normalizing to a provirus with wildtype Vpu (Vpu wt) (100% activity) and a Vpu-deficient provirus (ΔVpu) (0%). It should be noted that the raw output is inverted between the two assays: with tetherin, Vpu enhances infectivity, but with GaLV Env, Vpu inhibits infectivity.

Restriction is highly dependent on Vpu cytoplasmic tail, but not transmembrane region

Previous studies have demonstrated that Vpu's transmembrane domain (TMD) and cytoplasmic tail (CT) promote tetherin antagonism while only the Vpu CT has been identified for GaLV Env restriction (52, 169, 252, 282). VpuRD, a transmembrane "scrambled" mutant, is known to fully restrict CD4, but is ineffectual against tetherin (252). However, there have been conflicting reports about the importance of the TMD in CD4 restriction, with some studies suggesting a role of a conserved tryptophan (W22) in the C-terminal region (173, 279). We therefore sought to further investigate the role of Vpu's TMD by employing two previously described TMD mutants: VpuRD and W22L (173, 251-252). We introduced both of these mutants into our proviral system and tested their activity against tetherin and GaLV Env (Figure 4-2). As previously reported, both VpuRD and W22 mutants had decreased activity against tetherin (264, 282-283). However, both mutants exhibited wildtype activity against GaLV Env. In addition, we also included serine to alanine mutations at positions 53, 57. These serines are highly conserved and have been previously reported to be essential in tetherin and CD4

downmodulation (256, 282). As expected, the serines are important in downmodulation of both targets, presumably through their ability to mediate β-TrCP activity.

Vpu localization restricts antagonism of tetherin and GaLV Env

The subcellular location where CD4 and tetherin are targeted appears to be distinct. While action against CD4 has been reported to be exclusively in the RER, action against tetherin is generally believed to occur in a post-ER compartment (75, 175, 244, 263, 284). Previous studies demonstrated that Vpu retention in the RER by the addition of a putative retrieval motif prevents downmodulation of tetherin at the PM (283-284). We found that placement of the KKDQ ER-retention motif on the C-terminus of Vpu, exactly as previously described (284), reduced its ability to restrict either target, though the effect on tetherin restriction was more severe. These data are consistent with direct or indirect interactions between Vpu and both target proteins in a post-ER region.

Conserved amino acid features in Vpu cytoplasmic tail are required for activity

Next, we generated truncation mutations in Vpu to determine the minimal sequence required for modulation of the two targets in this system. For both tetherin and GaLV Env, truncation beyond 13 C-terminal amino acids (Δ13) resulted in a decrease in Vpu function, although for tetherin this decrease was progressive (Figure 4-2). To identify critical regions upstream of Δ13, we mutated two residues at a time to alanine and assayed for activity. For both targets, Vpu was most sensitive to mutations within

the conserved hinge region while upstream regions were less sensitive (Figure 4-3A). Unlike reported findings for BH10 Vpu R30A,K31A (75), mutations located within the YRKIL trafficking motif, we did not observe a decrease in infectivity in the presence of tetherin with our HXB2 Vpu system. Although both are subtype B, variances in amino acid sequence between the Vpus may explain differences.

We then sought to identify specific amino acids required in the CT by scanning individual point mutants through substitution of alanine for individual amino acids, with the exception of alanine which was substituted with serine. Interestingly, almost all amino acids within the Vpu-hinge region between amino acids 51-60, not solely the serines 53, 57, were sensitive to disruption (Figure 4-3B). A recent report suggested that a putative trafficking motif, EXXXLV, located between residues 60-65 is required for tetherin antagonism (146). In agreement with this report, we found that E60A disrupted tetherin activity, but the effects of L64A, and V65A alone were more modest. These results demonstrate Vpu's requirement for conservation of the hinge region for antagonism of two distinct protein targets. Because alanine substitution should not affect physical accessibility of the hinge region by proteins such as β-TrCP, we presume that modification of the conserved features, such as the acidic amino acids, disrupts recognition of Vpu by cellular factors or Vpu's ability to interact with targets.

DISCUSSION

Here we have identified shared critical features in Vpu required for restriction of two distinct proteins, tetherin and the glycoprotein GaLV Env. With the exception of the TMD region, Vpu requires similar features to counteract both targets. Our Vpu screen raises the question: why are similar features in Vpu required for modulation of two disparate target proteins? We propose that Vpu utilizes multiple regions for three somewhat overlapping steps in both restriction pathways: i) retention through interaction, ii) modification and redirection, and iii) degradation. In the case of tetherin, interaction occurs between the TMDs and for CD4 interaction occurs in the CTs and is absolutely required for antagonism (74, 112, 240, 252, 283). The importance of TMD interactions is highly evident in the evolution of species and subtype specificity of Vpu antagonism of tetherin (138, 248, 284). In the second step, we postulate that Vpu's CT-hinge region is required for both tetherin and GaLV Env modification and redirection. The hinge region likely represents a collective β-TrCP recognition motif, with serines housed within a conserved acidic stretch of amino acids. How Vpu modifies and subsequently redirects targets is not yet fully understood, although emerging data suggests a role of ubiquitination of both tetherin and CD4. While CD4 is polyubiquitinated, it is currently unclear whether tetherin is multiply monoubiquitinated or polyubiquitinated, hallmarks of redirection for lysosomal or proteasomal degradation, respectively (93, 175, 215, 280). In the final step of restriction, degradation of targets may occur. CD4 is directed for degradation through ERAD-proteasomal targeting (29, 48, 178, 251, 297, 300), However, the role of degradation for tetherin is unclear, with some data suggesting lysosomal (68, 93, 187) or proteasomal degradation (176). Interestingly, although

tetherin restriction can occur independently of the degradation, possibly through retention-based interactions, recent work demonstrates a significant role for lysosomal degradation of newly synthesized tetherin (72). We suspect that degradation may represent a late stage in restriction and may not be required until available Vpu becomes saturated.

Through our systematic alanine mutagenic library of the Vpu cytoplasmic tail, we identified specific amino acids contributing to the antagonism of two distinct targets, tetherin and a CD4 analogue, GaLV Env. Interestingly, we demonstrated a role for multiple amino acids within the CT hinge region and the importance of Vpu localization in restriction. Altogether our findings, along with other mutagenic Vpu studies, suggest that Vpu has unique regions mediating interaction with targets, while it uses conserved features within the CT to ultimately redirect and potentially degrade target proteins.

Acknowledgements

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Figure 4-1. **Schematics of HIV-1 proviral construct and experimental assay**

(A) HIV-1 HXB2 proviral construct with E2Crimson reporter showing enlargement of Vpu schematic outlining critical features in Vpu. Dotted outline predicted α-helices (103), bold script indicates the hinge region and underlined script highlights phosphorylated serines at positions 53,57. (B) For tetherin assays, 293FT cells (Invitrogen) were transfected with the following expression constructs: provirus (425 ng) and VSV-G (25 ng) with or without 12.5 ng of tetherin (kindly provided by P. Bieniasz) in a total of 500 ng. For GaLV Env assays, cells received 500 ng provirus, 25 ng of RSV Env ΔCT, and 475 ng MLV/GaLV Env (GaLV Env) (116, 169). At 48hr post-transfection, media was frozen overnight and used to infect target cells. Infected cells were fixed and analyzed by FACS on an Accuri flow cytometer. Flow plots illustrate typical data output for positive controls.

Figure 4-1: Schematics of HIV-1 proviral construct and experimental assay

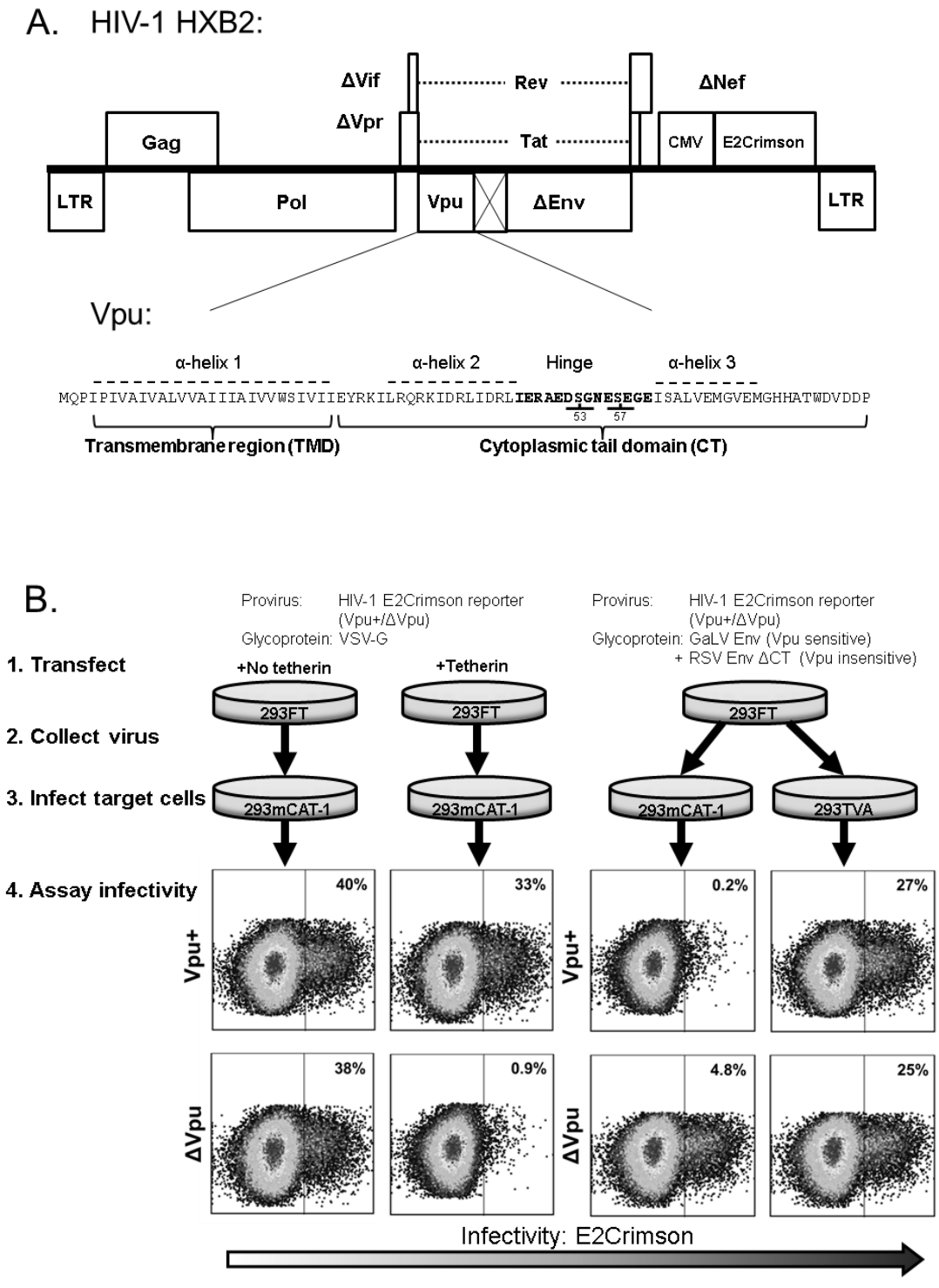


Figure 4-2: **Features required for Vpu-mediated antagonism of targets**

Tetherin (dark bars) and GaLV Env (light bars). (Top) Location of VpuRD, W22L (bold), critical serines 53, 57 (underline) and truncations (arrows) are noted in the Vpu schematic. (Bottom) Relative Vpu activity is shown as mean averages (n=3-4, ±SE) calculated by normalizing infectious units per ml for each mutant Vpu relative to Vpu wildtype (Vpu wt) (100%) and no Vpu (ΔVpu) (0%).

Figure 4-2: Features required for Vpu-mediated antagonism of targets

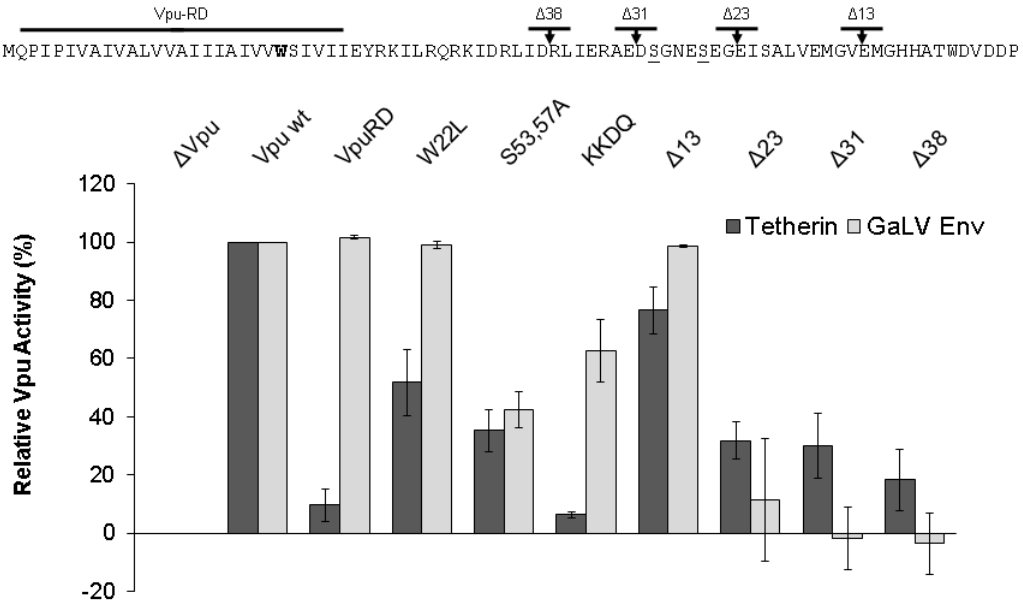
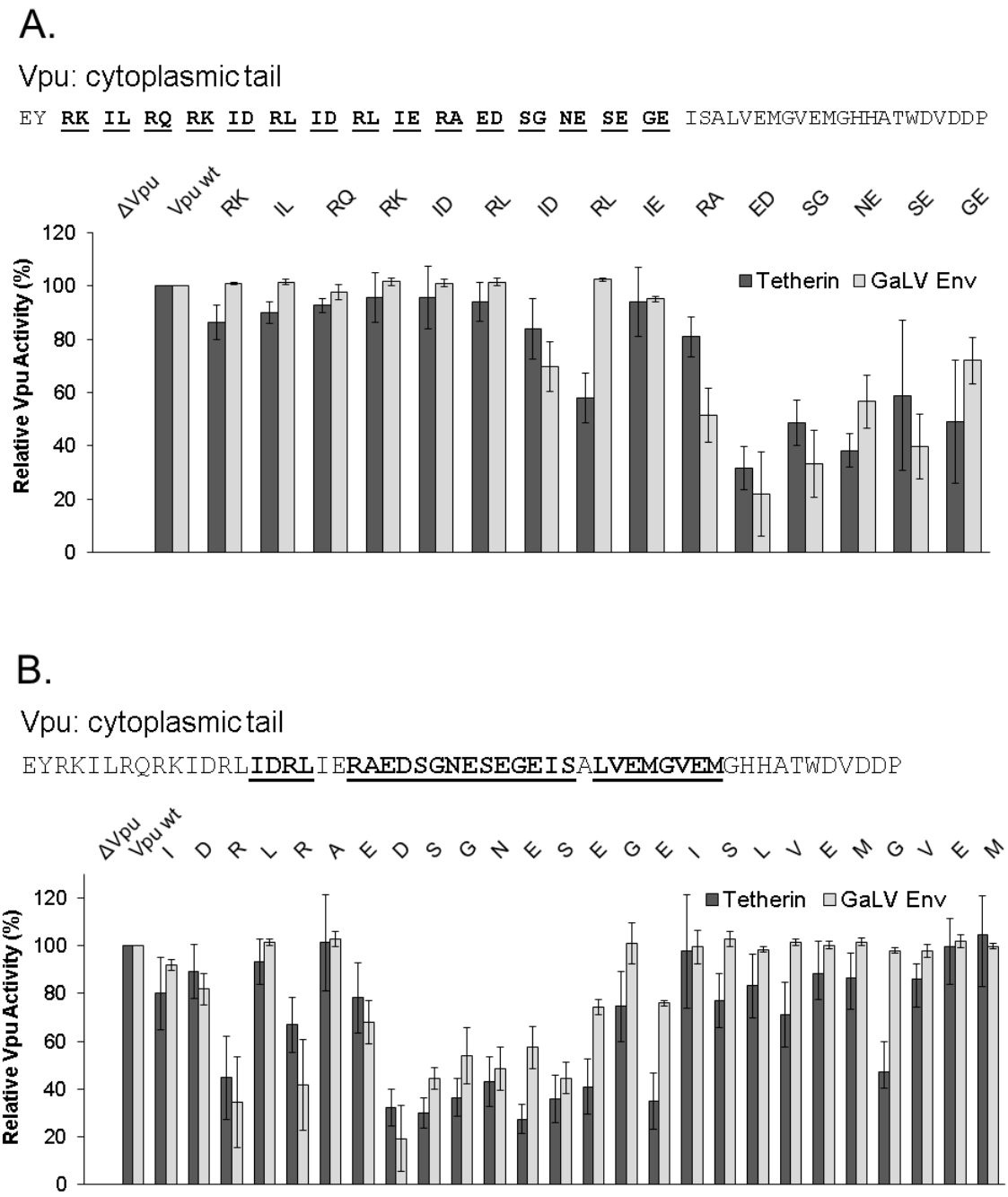


Figure 4-3. **Alanine mutagenic scan of Vpu reveals antagonistic regions for downmodulation of tetherin and GaLV Env**

Tetherin (dark bars) and GaLV Env (light bars). Amino acids were mutated to alanine, with the exception of alanine which was mutated to serine (A) A double alanine-mutagenic scan was performed on the cytoplasmic tail region of Vpu (double mutations, underlined). (B) An individual amino acid alanine scan was analyzed for amino acids identified in the double-alanine scan (bold, underlined) and relative Vpu activity was measured. Relative Vpu activity is shown are mean averages (n=3-4, $\pm$SE) calculated by normalizing infectious units per ml for each mutant Vpu relative to Vpu wildtype (Vpu wt) (100%) and no Vpu (ΔVpu) (0%).

Figure 4-3: Alanine mutagenic scan of Vpu reveals antagonistic regions for downmodulation of tetherin and GaLV Env



V. Hydrophobicity of membrane spanning domain of Env dictates fusogenicity, but not Env recruitment to viral particles

Sanath Kumar Janaka, Devon A Gregory and Marc C Johnson

Abstract

Non- native viral glycoproteins, including Friend murine leukemia virus (F-MLV) envelope (Env) are actively recruited to HIV-1 assembly sites by an unknown mechanism. Because HIV-1 Gag is a membrane binding protein that could modify the local membrane environment, we wanted to examine the contribution of the Env membrane spanning domain (MSD) in MLV Env recruitment to budding viruses. To examine the importance of MSD hydrophobicity in Env recruitment, a series of mutant MLV Env genes were constructed that added or deleted one, two, or three leucines in the MSD. -1L, -2L, -3L, +1L and +2L mutations did not prevent Env incorporation into HIV-1 particles, but abolished fusogenic activity and infectious particle production. Surprisingly, +3L Env was able to produce infectious particles and was constitutively fusogenic. However, when the CTD of Env was deleted, the MSD mutants partially regained the ability to produce infectious particles. Further mutational analyses revealed that the first 10 amino acids of the CTD is a critical regulator of infectivity. A similar phenotype was observed in HIV-1 Env upon addition of leucines in the MSD, with +1 and +2 Leucine mutations greatly reducing Env activity, but +3 Leucines behaving

similar to wildtype. Unlike MLV Env (+1L, +2L), HIV-1 Env (+1L, +2L) infectivity was not restored by deletion of the CTD. We hypothesize that the fusion incompetency observed with many of the mutants arises from the torsion on the Env upon addition or deletion of the leucines, except in the case of +3L Env. With the +3L Env, an extra helical turn would be added in the MSD and this may be sufficient promote fusogenic activity.

Introduction

Enveloped viruses assemble by the confluence of structural proteins, genetic material and surface glycoproteins. Glycoprotein-deficient viruses have the potential to be complemented by diverse, non-native glycoproteins in a process termed pseudotyping [reviewed in (60, 121, 182, 214)]. The molecular mechanisms underlying the recruitment of foreign glycoproteins to budding viral particles are not understood. However, such pseudotyped viruses can be used as a gene delivery tool to direct viruses to a specific cell type (12, 65, 95, 134, 181, 213).

Retroviral Env glycoproteins are produced as precursors that trimerize in the endoplasmic reticulum (ER). Subsequently, the precursor protein is cleaved into its constituent sub-domains, namely, the surface subunit (SU) and transmembrane subunit (TM) by furin or a furin-like-protease. The mature Env is thus a trimer of heterodimers, composed of the SU, containing the receptor binding domain and TM containing the fusion peptide. SU and TM are held together by an inter-subunit disulphide bond in the case of alpha-, gamma-, and delta-retroviruses (80, 122, 152, 208, 223, 225); and non-covalently associated in the case of lenti- and beta-retroviruses (102, 143). Upon receptor binding by SU, several conformational changes take place in Env, promoting co-receptor binding and/or accession of the host cell membrane by the fusion peptide in TM (76). A functional Env can deliver the viral components to the host cell, post fusion.. In the case of gammaretroviruses, fusion is controlled by the C-terminal cytoplasmic tail domain (CTD) of Env and isomerization of disulphide bond between SU and TM (164, 289). The

C-terminus of these glycoproteins contains a short peptide (R-peptide) that is cleaved by the viral protease during or shortly after viral assembly. The presence of the R-peptide at the C-terminus of Env prevents fusogenic capability of the Env (145, 157, 164, 234-235, 276, 306). Cleavage of the R-peptide allows for receptor dependent fusion of the Env to the target cell (100, 235). Recent cryo-EM imaging data suggests that the CTD of MLV Env holds the MLV Env ectodomain in a tight conformation, but upon cleavage of the R-peptide, the TM legs are splayed and this allows fusogenic activation of Env (165).

Glycoprotein acquisition by retroviral particles is not well understood and the physical factors that contribute to this recruitment have not been defined, but multiple viral glycoproteins have been reported to efficiently pseudotype retroviral particles [reviewed in (60, 121, 214)]. Not every glycoprotein-virus pair results in infectious particles, but the recruitment of such a variety of surface proteins would indicate the use of a common mechanism by the virus to obtain its compatible glycoprotein. Some reports indicate a direct interaction between the N-terminal matrix (MA) of Gag and the CTD of Env may promote packaging of Env into viral particles (88, 177, 194, 211). In the case of HIV-1, several studies have shown the MA domain of HIV-1 Gag to be required for HIV-1 Env incorporation into budding viral particles. For instance, compensatory mutations in MA of HIV-1 have been selected for when paired with incorporation defective HIV-1 Env mutants (194), suggesting a direct interaction between HIV-1 MA and the HIV-1 Env CTD. However, infectious HIV-1 viral particles can still be produced when the entire CTD of HIV-1 Env is deleted, and in some cases such CTD truncations are naturally selected for when lentiviruses are passaged in tissue culture (42, 129, 141-142,

261-262). Furthermore, we have demonstrated previously that MLV Env with its CTD deleted is efficiently recruited to HIV-1 assembly sites (168). Contrasting evidence has been presented by Muranyi et al., and they have recently shown that in the case of HIV-1 Env, truncation of the CTD leads to non- specific recruitment of Env to Gag budding sites (196) Thus, direct interactions between MA and the glycoprotein CTD are not the sole factors dictating glycoprotein recruitment to viral assembly sites (10, 88, 177, 194, 211). To understand Env recruitment further, we chose to examine the contribution of the MSD in MLV Env to glycoprotein trafficking and fusogenicity. We hypothesized that the hydrophobicity of the MSD of Env would contribute to the trafficking of Env to viral particles. We chose to focus on a stretch of ten continuous hydrophobic amino acids in the MLV Env MSD (Figure 5-1). To understand if this hydrophobic region in the MSD of Env contributes to recruitment of the glycoprotein to viral particles, we engineered the addition or deletion of 1, 2 or 3 leucines to modulate the hydrophobicity of the MSD. Biochemical and infectivity assays were performed to understand recruitment of Env; cell-to-cell fusion assays were performed to understand the effect of the mutations on the fusogenicity of Env.

Materials and Methods:

Plasmids

The F-MLV Env and YFP tagged F-MLV Env expression constructs were a kind gift from Dr. Walter Mothes (Yale Unviersity). The Leucine addition/ deletion mutations in the MSD in F-MLV Env were created by inserting linkers carrying the appropriate

mutations in the region of the Env coding DNA between BclI and ClaI sites in the YFP tagged Env construct. The corresponding untagged Env constructs were created by digestion and ligation of the PasI to EcoRI segment to the WT F-MLV Env construct. Constructs expressing the truncated form of the F-MLV Env were created by introducing stop codons at the appropriate positions in the linker replacing the fragment between ClaI and EcoRI. Alanine scanning mutations in the context of the Δ21 construct was created by linkers carrying the appropriate mutations and replacement of the fragment between ClaI and EcoRI. The HA tagged F-MLV Env was created by replacement of the DNA fragment encoding YFP with that encoding the HA tag (YPYDVPDYA). The B- clade consensus, codon optimized pcDNA 3.1 HIV-1 gp160 expression construct was a kind gift from Dr. Beatrice Hahn (University of Pennsylvania). Leucine addition mutants in the MSD of HIV-1 Env were introduced by overlap extension PCR, with the primers carrying the appropriate mutations and replacement of the fragment of DNA between PasI and BamHI. HIV-1 Env CTD truncation mutants were created by introducing the stop codon after RVRQGY, a truncation removing 144 amino acids in the HIV-1 Env WT Env. Primers carrying the mutation were used in PCR amplification and replacement of the fragment between BamHI and BsrGI.

NL4-3 derived HIV-CMV-GFP was kindly provided by Vineet KewalRamani (National Cancer Institute-Frederick). This proviral vector lacks the genes encoding Vif, Vpr, Vpu, Nef and Env and has a CMV immediate-early promoter driven GFP in the place of Nef. A vector expressing HIV Gag with the late domain (PTAP) changed to alanines (HIV Gag PTAP→AAAA) was previously described (132).

The lentiviral vector with a CMV driven, reverse intron-interrupted Gaussia Luciferase was a gift from David Derse. The reporter gene with the intron was amplified and dropped into the HIV-CMV-GFP vector in the appropriate orientation by PCR and replacement of the DNA fragment between NotI and XhoI, encoding the CMV driven GFP. Only the infected cell, but not the transfected cell, can produce an intact luciferase protein, leading to signal specifically from an infection (8, 183).

The tet-off expression plasmid was created by introducing the gene encoding the tTA (Tet-Off) protein into a pQCXIP vector (Clontech) between NotI and BamHI sites, downstream of the CMV immediate early promoter. The retroviral transfer vector, retro-tight-X-hygro from Clontech was used to create a TRE driven *Gaussia* luciferase (GLuc) inducible expression construct (TRE-GLuc). Specifically, the gene coding for the Luciferase protein from the marine organism, *Gaussia princeps*, was introduced between BamHI and NotI sites downstream of the Tetracycline response element (TRE).

Cell culture

The 293FT cell line was obtained from Invitrogen. The cell line expressing the ecotropic F-MLV Env receptor, 293T mCAT-1, was kindly provided by Walther Mothes. The 293T TVA cell line expressing the receptor for Rous sarcoma virus (RSV) Env was provided by John Young (Scripps Research Institute) (156). TZM-bl cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

The following procedure was used to create 293T mCAT-1 cells with a stably integrated, tet-off inducible Gaussia Luciferase reporter system. 293FT cells were transfected with constructs expressing MLV GagPol, TRE-Gluc (Also codes for hygromycin resistance gene) and VSV-G. The supernatant from this transfection was used to infect 293T mCAT-1 cells. The cells were selected for Hygromycin expression. Further, clonal isolates were obtained by following a procedure of limiting dilution. Six clonal populations were tested. Two cell lines were selected for further assays based on level of induction of the reporter and the presence of the mCAT-1 receptor.

All HEK based cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418. In phases of cell culture involving transfection or transduction, G418 was not added to the medium. TZM-bl cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine and 1% MEM vitamins.

Infectivity assay

293FT cells were transfected with 500ng of HIV-CMV-GFP and 500ng of the wildtype (WT) or mutant F-MLV Envs using 4μg of Polyethylenimine (PEI) per microgram of DNA (31). For transfections with HIV-1 Env and its mutants, 950 ng of HIV- CMV-GFP and 50 ng of the WT or mutant HIV-1 Envs were transfected into 293 FT cells. The media was changed 6-12 h later to remove the residual transfection reagent. Supernatant

was collected 24 h after the media were exchanged and then frozen at -80^{o}C for at least 2 h to lyse any cells in the supernatant. The supernatant was thawed in a 37^{o}C water bath and spun at 1500 g for 10 min to pellet any cells or cell debris. 500µl of the supernatant was applied to fresh 293T mCAT-1 cells in a 12 well plate for transfections with MLV Env. For transfections with HIV Env, 500µl of the supernatant was added to TZM-Bl cells along with Hexadimethrine Bromide to a final concentration of 80µg/ml (56). Cells were collected 48 h later, fixed with 4% paraformaldehyde, and analyzed by the Accuri C6 flow cytometer system. Data were analyzed by Accuri C6 software.

Western Blots

Transfections for western blots were performed as described for infectivity assays. HA tagged F-MLV Env derivatives were used for analysis by western blotting. Viral samples were pelleted through a 20% sucrose cushion. The residual serum was removed by washing the pellet with PBS. The pellets were resuspended in 2X SDS-PAGE loading buffer and the equivalent of 1ml of viral supernatant was analyzed by 10% discontinuous SDS-PAGE. Cell samples were prepared in 1X SDS-PAGE loading buffer and 5-10% of the total amount of cells was analyzed in parallel with the viral supernatants. Proteins were transferred manually onto a 0.45 µm PVDF membrane. The membrane was blocked with 1% non- fat dried milk in PBS-Tween20 and probed with mouse anti HA antibody diluted 1:1000 (Sigma) and mouse anti-HIV p24 hybridoma medium diluted 1:500 (obtained through the AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH: HIV-1 p24 hybridoma [183-H12-5C]) from Bruce Chesebro (49).

Blots were then probed with horseradish peroxidase conjugated anti-mouse antibody (Sigma) diluted 1:10000. Luminata Classico western HRP substrate from Millipore was used for visualization of the membranes on a chemiluminescence image analyzer, LAS3000 from Fujifilm. Blots were also probed with IR dye 700DX anti mouse IgG and visualized using Odyssey infrared imaging system from LI-COR biosciences.

Viral Capture Assay

293FT cells were transfected with 900ng of HIV provirus with the intron interrupted Guassia Luciferase reporter, 25 ng of Rous Sarcoma Virus (RSV) Env ΔCTD and 75 ng of YFP tagged F-MLV Env or its mutants as described previously. 96 well ELISA plates (Corning) were coated with polyclonal anti-GFP antibody from rabbit diluted 1:5000 in ELISA coating buffer, overnight at 4^0C. The antibody was removed and replaced with ELISA blocking buffer for 1 h at room temperature. 50μl of viral samples were incubated in the wells after removal of the blocking buffer for 4 h. The wells were then washed with PBS and 10000 cells of 293T mCAT-1 or 293T TVA cell lines were added to the wells. The experiments were done with or without antibody with both cell lines. Direct transductions of the above mentioned cell lines were also performed.

Scanning Electron Microscopy

Correlative SEM was performed as previously described (132, 151). Briefly, 293T mCAT-1 cells were plated onto glass coverslips with a thin-layer gold coat in a grid

pattern and transfected with 800ng of HIV Gag PTAP→AAAA and 200ng of YFP-tagged MLV Env or one of its mutants using PEI as described earlier. Transfected cells were mapped by fluorescence microscopy. Anti-GFP 20 antibody (G6539; Sigma) was used to label YFP-tagged MLV Env. Gold-labeled anti-mouse secondary was obtained from Jackson Laboratories (catalog no. 115-205-146). After critical point drying and carbon evaporation coating, samples were imaged with a Hitachi S4700 FE SEM at the University of Missouri Electron Microscopy Core Facility.

Cell-to-cell fusion assay

293FT cells were transfected with 500 ng of tet-off expression plasmid and 500 ng of an Env expression construct. The media was changed 6-12 h post transfection to remove any residual transfection reagent. 100000 of the transfected cells were co-cultured with an equal number of 293T mCAT-1 TRE G Luc cells for 48h. 20µl of the supernatant on the co-cultured cells were assayed in duplicate for G Luc with 50µl of 10□M coelenterazine in 0.1M Tris, pH 7.4 and 0.3M sodium ascorbate. Each experiment was performed along with WT F-MLV Env for a negative control, Filler DNA in the place of Env for another negative control and also with F-MLV Env lacking the R-peptide (F-MLV ΔR) as a positive control. All data were normalized to F-MLV ΔR Env fusogenicity.

Results

Assembly of Gag at the inner leaflet of the plasma membrane has been proposed to modify the local lipid composition of the bilayer and this may promote further recruitment of transmembrane proteins- either native viral or non-native glycoproteins (144, 206). This hypothesis would indicate the MSD of Env plays a critical role in the recruitment process. The MSD of F-MLV Env contains a string of ten uninterrupted highly hydrophobic amino acids (Figure 5-1). While MSDs are always composed of hydrophobic amino acids, this continuous stretch of exclusively hydrophobic amino acids represents the extreme of hydrophobic domains.

Addition or removal of leucines in the MSD of F-MLV Env affects the production of infectious viral particles

We hypothesized that the hydrophobic region in the F-MLV Env would contribute to its recruitment to viral particles. To test this hypothesis, the MSD was mutated to contain 1, 2 or 3 fewer or additional leucines (termed -3L, -2L, -1L, +1L, +2L, +3L, respectively; WT Env is termed '0L') within the hydrophobic block of amino acids, highlighted in Figure 1. Leucine, being a hydrophobic amino acid, would be expected to change the net hydrophobicity of the MSD in addition to changing the MSD length. Initially, infectivity of an Env-defective HIV-1 provirus (HIV-CMV-GFP) co-transfected with these Envs was tested. We expected many of these Env clones, particularly those with large changes, to be non- functional. Surprisingly, functionality among these mutants did not appear to be correlated with hydrophobicity of the MSD. Only WT Env (0L) and +3L

Env were able to generate infectious particles with HIV-1 cores, all others were non-functional in infectivity assays (Figure 5-2A). In addition to this approach, we also altered the hydrophobicity of the MLV Env by replacement of three leucines in the hydrophobic core of the MSD to three isoleucines (same hydrophobicity) or three alanines (decreased hydrophobicity). In such cases, where the length of the MSD was not affected and hydrophobicity was altered, the infectivity was equivalent to WT F-MLV Env and again showed that the hydrophobicity of the Env MSD did not determine Env recruitment (Figure 5-S1).

MSDs of transmembrane proteins usually assume a helical conformation and secondary structure prediction algorithms predict the F-MLV Env MSD to be helical (36, 70). Since addition of three Leucines would introduce a turn in the helix, this could explain why this was the only mutant with significant activity. To explore this further, two additional mutants with Four (+4L) or five (+5L) Leucines added to the MSD was assayed for infectivity (Figure 5-S2). While +4L mutant was non-infectious, +5L mutation partially restored infectivity to the Env, suggesting that the helical orientation of the MSD contributes to Env functionality. Because +4L and +5L did not regain function, the remainder of the study focused on mutant -3L to +3L.

Leucine additions or deletions do not affect viral incorporation of Env

To determine if the changes in the transmembrane domain affect the incorporation of Env into viral particles, western blotting was carried out on cell lysate and viral supernatant

from cells expressing each mutant Env along with HIV-CMV-GFP. The HA epitope tag (YPYDVPDYA) was introduced into the mutant Envs in the variable region of the proline rich region, a region in MLV Env where insertions of peptides are tolerated without any effect on Env function (79, 241, 302). F-MLV Env is produced as a gp85 precursor and processed by a cellular protease into gp70 (SU) and p15 (TM)(201). In the case of mutants -1L, -2L, -3L, and +2L there was a noticeable reduction in amount of processed Env. However, all seven of the Env proteins were incorporated into viral particles at equivalent levels. Unexpectedly, transfected cells expressing mutants -1, -2, and -3 released some processed and unprocessed Env in a pelletable form even in the absence of the structural proteins. Addition of HIV-1 GagPol to the transfection increases the amount of both processed and unprocessed Env released into the media (Figure 5-2B).

To rule out any ambiguities in the incorporation of Env into viral particles, we performed an alternative infectivity based assay to quantify Env incorporation into viral particles. For this assay, outlined in Figure 5-3A, particles are produced in the presence of one of the mutant MLV Env proteins as well as Rous sarcoma virus Env ΔCTD, which we demonstrated previously to be randomly incorporated into HIV-1 particles (132). These particles are adhered to a tissue culture plate with an antibody against MLV Env and unbound virus is washed away. Bound virus is then overlaid with 293-TVA cells, which are susceptible to fusion with RSV Env, but not MLV Env. Thus, infectivity of the 293-TVA cells reflects incorporation of MLV Env into particles.

An MLV Env construct with YFP engineered into the proline rich region was used for this assay because we have found that the YFP tag in MLV Env works well for viral capture with a polyclonal anti-GFP antibody. Each of the mutants was engineered with YFP and assayed to see if their phenotype was altered by the presence of YFP. For this assay an Env-defective HIV-1 provirus containing Gaussia luciferase with a reverse intron (HIV-CMV-iGLuc) was used (8, 183). As expected, infectivity on cells with the receptor for MLV Env (293T mCAT-1) was highest with MLV Env 0L and +3L (Figure 5-3B, straight infection). With this assay it was apparent that some infectivity occurred with all of the Env mutants, but -1L, -2L, -3L, +1L, and +2L were all reduced about 100-fold compared to wildtype and +3L. This infectivity with the YFP-tagged mutants followed the same pattern as the untagged Envs (Compare Figure 5-2A and Figure 5-3B). Next we tested if the viral particles could be adhered with the GFP antibody. Virus was adhered to the plate and washed prior to infection as outlined in Figure 5-3A. GLuc signal equivalent to that from the straight infection was observed with each of the mutants, and the signal in each case was greatly diminished in controls with no antibody. Note, the infectivity in the control wells with no antibody containing the 0L and +3L Envs appeared high compared to the poorly functioning glycoproteins, but this signal was still 100-fold lower than the equivalent wells that did contain antibody. Next we tested infectivity on cells susceptible to infection with the RSV Env to determine if any of the mutant glycoproteins were incorporated and capable of facilitating viral capture. Direct transduction of 293T TVA cells with the viral supernatant showed the presence of infectious viral particles in the supernatant from all transfections. Infectivity by virus capture against the YFP tag followed by infection on 293 TVAs was comparable in all

samples containing the YFP tagged F-MLV Env or a mutant thereof. This experiment confirms that leucine mutants in F-MLV Env are incorporated to similar levels into viral particles.

Several cellular proteins are incorporated into HIV particles by virtue of their abundance at the site of viral budding (210), which may also be the case with non-native viral glycoproteins. MLV Env recruitment to viral particles however has been shown to be specific using a correlative SEM imaging technique (151, 168). Since MSD mutants may change the trafficking and characteristics of the Env, we wanted to understand if the mutation affected the specific recruitment phenotype. In 293T mCAT-1 cells, when late domain defective, HIV Gag PTAP→AAAA was expressed, YFP-tagged +1L MLV Env was specifically recruited to viral budding sites (Figure 5-S3). The hydrophobicity changes in Env therefore do not affect recruitment of MLV Env to HIV budding sites.

Addition/deletion of Leucines in MSD of F-MLV glycoprotein affects fusogenecity of Env

Because incorporation of the Leucine insertions and deletions did not affect incorporation into particles, we questioned why they were non-functional. To understand the infectivity phenotype with these mutants, we tested the fusogenicity of each mutant Env. Because Gammaretroviral Env proteins are fusogenically inactive prior to cleavage of the R-peptide, each of the Env mutants was engineered to have the R-peptide removed and the fusogenicity of each mutant (with or without the R-peptide) was assayed.

Briefly, each mutant Env was transfected along with a tet-off expression construct into 293 FTs. 24 h post transfection, 100,000 of the transfected cells were co-cultured with 100,000 293T mCAT-1 cells expressing TRE driven GLuc (See materials and methods). If Env is fusogenic, the transfected cells and the receptor expressing cells fuse, resulting in a tet-off dependent induction of GLuc. The extent of induction of the reporter relative to the WT Env and the ΔR Env indicates the fusogenicity of the Env. The mutant full length (FL) Envs, regardless of mutations in the MSD were found to be non-fusogenic by this assay, with the exception of +3L Env. The +3L Env is constitutively fusogenically active despite the presence of the R-peptide (Figure 5-4A). Upon deletion of the R-peptide, the 0L and +3L Envs were the most fusogenic, but most of the ΔR Env mutants displayed some fusogenic activity. Because the ΔR mutants were at least partially fusion capable, we tested this panel of mutants for the ability to form infectious particles. With the exception of the wild type and +3L mutants, the leucine mutants remained non-infectious despite having the R-peptide deleted (Figure 5-4B**)**. Finally, we tested whether there are additional sequences upstream of the R-peptide that act as negative regulators of fusion by engineering the leucine mutants with the majority of their cytoplasmic tail deleted (Δ25 mutants). In each case, the infectivity with the truncated Env was higher compared to the infectivity with the respective FL Envs (compare Figure 5-4B and Figure 5-2A). The mutant -2L Δ25 Env did not produce a high level of infectious particles and it may relate to the level of the mature Env with this mutant as seen in the case of -2L FL Env (Figure 5-2B). Having thus implicated the CTD in this observed phenotype, we wanted to determine the sequences in the membrane proximal region of the CTD responsible for modulating infectivity in the MSD mutants.

Sequences in the CTD contributing to infectivity with MSD mutations in Env

While removal of most of the CTD (Δ25) restored infectivity to most of the MSD mutants, removal of the R-peptide did not. This suggests that the sequence just proximal to the MSD can modulate fusogenic activity. MLV Env is trimeric in the ectodomain and also is modeled to be trimeric in the CTD prior to R-peptide cleavage (80, 165, 276). A recent study by Loving et al. (165) demonstrated that the membrane proximal ectodomain of MLV Env is tightly packed, but in the absence of the R-peptide, the TM legs are splayed apart. These pieces of data led us to hypothesize that the CTDs in the MSD mutants form a tight trimer that prevents fusion unless the majority of the CTD is deleted. To identify the minimum portion of CTD that negatively regulated fusion of the MSD mutants, we created truncations in the 0L and +1L Env. These Envs are processed efficiently in the cell; they are incorporated into viral particles and are non-fusogenic with FL CTD. However, whereas the +0L Env can produce infectious particles regardless of its CTD, the +1 can only produce infectious particles if its CTD is deleted. Exploiting the singular difference in the infectivity with these two Envs, we explored the sequences required for fusion control and modulation of infectivity. Initially, truncations in the CTD removing 21, 22, 23 or 24 amino acids were made in the 0L and +1L Envs. The infectivity of HIV-1 particles with these Envs showed that removal of 23 C-terminal amino acids in the CTD of the +1L Env allowed infectious particle production (Figure 5-5A).

To test whether the amino acid sequences N-terminal to position 21 were involved in the modified infectivity and fusogenicity, amino acids between positions 21 and 29 were mutated to alanine as sequential double mutants or single point mutants in the context of an Env Δ21 mutant. Mutation of F28, either as a part of a double substitution or as a point substitution, reduces infectivity drastically to 0L Env Δ21 and +1L Env Δ21. Mutations of amino acids R24 and D25 restored infectious particle production to +1L Env Δ21, while other mutations in this section of eight amino acids did not change its phenotype (Figure 5-5B). Mutation of R24 or D25 alone has a less significant affect (Figure 5-5C). In the case of all other mutations, 0L Env is not affected in its capacity to produce infectious pseudotyped HIV-1 virus (Figure 5-5B and C). To test if the RD23, 24AA mutation was sufficient to restore Env function to the +1 Env in the context of the FL CTD, the mutation was recreated in the FL 0L and +1L Envs and tested for infectivity (Figure 5-5B). Mutation of these two residues was not sufficient to restore infectivity in the context of the FL CTD. In fact, mutation of these residues also drastically reduced infectivity of the +0 FL Env. Thus, these two residues are important contributors to Env regulation, but are not the only residues required to negatively regulate Env function.

HIV-1 Env MSD hydrophobicity does not dictate Env recruitment and infectivity modulation is independent of the CTD:

With the F-MLV Env, fusion regulation is achieved by sequences in the CTD and hence helical torsion of Env may affect the fusogenicity of the Env in a CTD dependent manner. While HIV-1 Env is activated for fusion upon HIV-1 protease activation and

viral maturation, there is no proteolytic cleavage of the Env itself (303-304). To understand if this infectivity phenotype was restricted to Envs processed by protease for fusion activation, we examined HIV-1 Env. Leucine addition mutations in the MSD HIV-1 Env were created and the infectivity was assayed in each case. Similar to MLV Env, addition of one or two leucines in the MSD prevented infectivity, while WT and +3L HIV-1 Env were infectious (Figure 5-6). Further, the CTD of each of the Leucine addition mutants in HIV-1 Env was deleted to understand the role of the CTD in this phenotype for a non-proteolytic fusion controlled Env. The HIV-1 Env CTD truncation mutants behaved similar to their FL counterparts indicating that although MSD hydrophobicity may affect function of Env, the CTD is not required for this modulation (Figure 5-6).

Discussion

We have shown previously the active recruitment of foreign glycoproteins to HIV-1 budding sites (132). Further studies into protein domains in MLV Env required for recruitment to HIV-1 assembly sites showed that the cytoplasmic tail domain was not required for specific recruitment (168). Furthermore, Gag domains contribute to selective recruitment of foreign glycoprotein to sites of viral egress and MA is dispensable for such Env recruitment (91). Gag being a membrane binding protein that modifies the lipid and protein environment in the membrane (144, 206), it may possibly recruit Env to viral budding sites through interactions with the MSD. This study evaluates the role of the hydrophobicity of Env MSD in Env recruitment to HIV-1 viral budding sites.

MSD Hydrophobicity does not affect Env recruitment

In contrast to our expectations, the hydrophobicity of the MSD did not appear to play a role in recruitment to viral particles (Figure 5-2A, Figure 5-6 and Figure 5-S1). Virion biochemical analysis and the Viral capture assay shows that the Env is incorporated into viral particles regardless of the mutation (Figure 5-2B and 3B). Further, all the mutant Envs are incorporated to comparable levels (Figure 5-3C, anti GFP (MLV Env) capture, infection on 293 TVAs). These data showed that the hydrophobicity of the Env is not a necessary factor in its recruitment to viral particles, although the hydrophobicity could affect other stages in the Env biogenesis, With the Leucine addition/deletion mutants, since the Env is present in viral particles, the only reason for its variation of infectivity could be the Env fusogenicity. Indeed, a test of cell-to-cell fusogenicity of the different mutant Envs showed that the variation in infectivity mirrored the fusion function of the different Envs (Figure 4A).

Helical phasing of Env MSD controls Env function

Evaluation of gp70 and gp85 in cells showed that removal of Leucines in the MSD reduced Env processing (Figure 5-2B) and was deleterious for Env function (Figure 5-4A). Addition of one or two leucines abolished fusogenicity of Env, while addition of three leucines made the Env constitutively fusogenic (Figure 5-4A). These data would be consistent with the helical conformation of the Env. The addition of a single amino acid would twist the orientation of the protein with an alpha helix by approximately 103^0. Addition of three or four leucines would effectively introduce one turn of the alpha helix

and hence would bring the protein back to the original orientation. However, single amino acid increments or decrements in the helix would introduce torsion in the structure and this may prevent the normal function of the protein, in this case the F-MLV Env. Having seen constitutive fusogenicity of the +3L Env, we considered two possibilities- 1) Addition of three leucines would introduce an extra turn of the helix in the MSD, which restored the phase of the helix, thus promoting a fusogenic Env trimer; 2) The addition of an extra turn of the helix/ three leucines in the MSD could possibly provide a stable intermediate for fusion and hence the fusion- restrictive sequences in the CTD are no longer required. If the helical torsion determines Env function, then addition of four or five leucines in the MSD would make the Env sub-optimal. On the other hand, if three extra leucines in the MSD are enough to support a fusogenically active Env, addition of further leucines will not affect the infectivity seen with the +3L Env. Infectivity with +4L and +5L F-MLV Env pointed that the former hypothesis is true i.e., helical torsion in the Env reduces its functional capabilities, and the presence of an extra helical turn in MSD alone cannot promote fusogenic activation.

Acknowledgements

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Kewalramani at NCI. HIV Env expression construct was a kind gift from Dr. Beatrice Hahn and TZM-bl cells were obtained from NIH AIDS reference reagent program.

Figure 5-1: **Schematic of F-MLV Env**

Sequences in the membrane spanning domain (MSD) and cytoplasmic tail domain (CTD) are shown. The leucine rich region that has been modified with leucine frameshift mutations has been highlighted.

Figure 5-1: Schematic of F-MLV Env

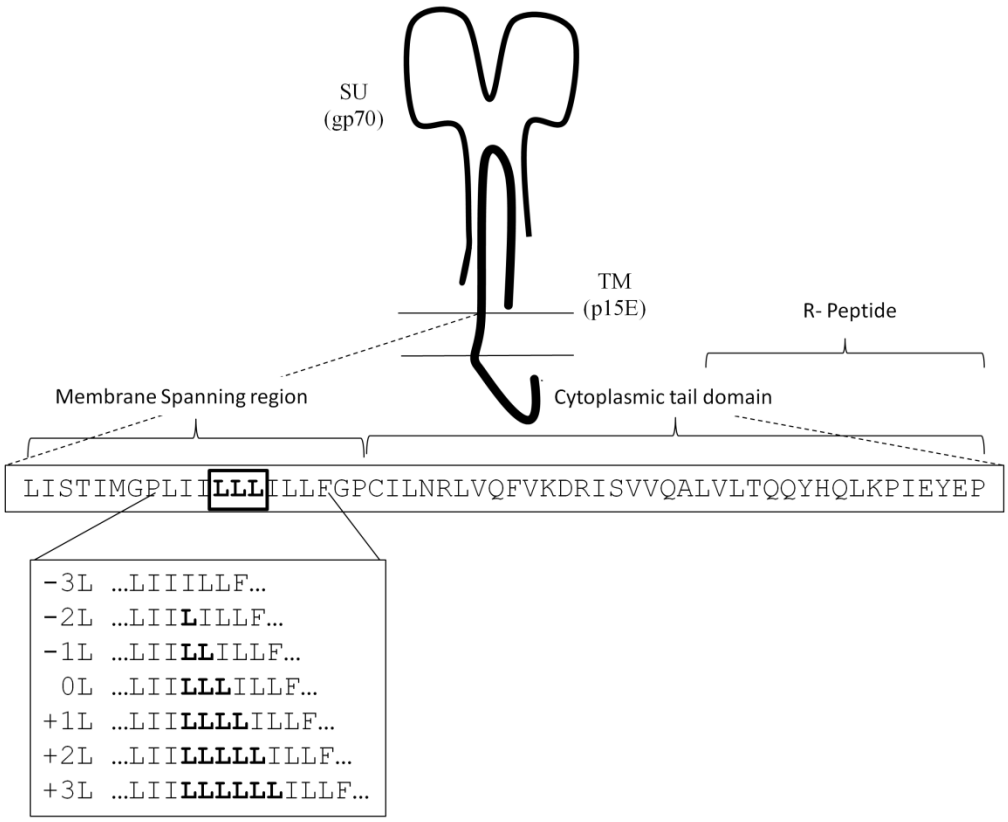


Figure 5-2: **Hydrophobicity of MSD does not affect Env incorporation in viral particles**

(A)Infectivity relative to WT MLV Env of the various mutants are shown. Data shown here are an average of at least three experiments. Bars indicate the SD in the experiments. (B) 293 FTs transfected with HIV-CMV-GFP and HA tagged Env constructs were used in biochemical analyses of cells and viral particles. Representative image shows antibody probing against HA (Env) and p24 in cell lysates and viral pellets

Figure 5-2: Hydrophobicity of MSD does not affect Env incorporation in viral particles

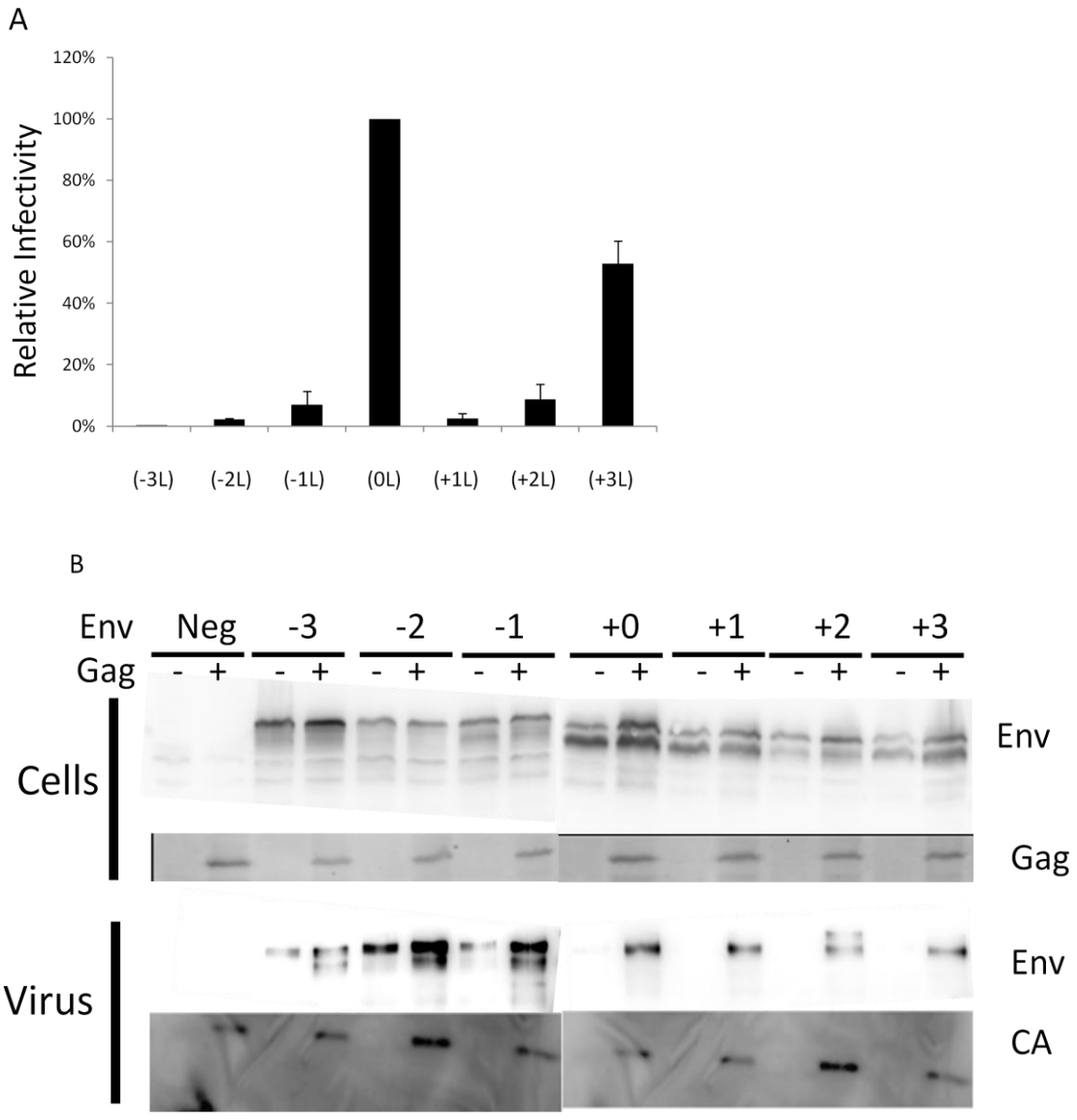
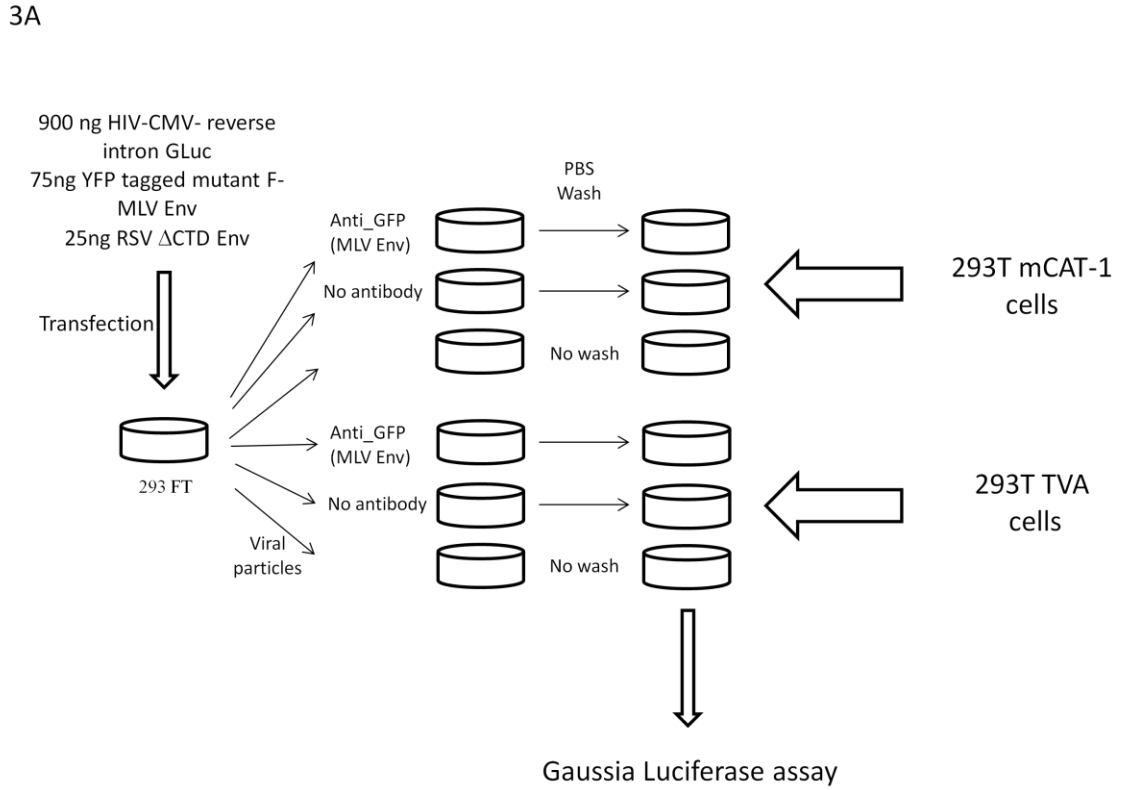


Figure 5-3: **Infectious viral particle production is not affected by the presence of the leucine frameshift mutant F-MLV Envs**

(A) Schematic of the virus capture assay. (B)Infectivity of viral particles pseudotyped with the indicated mutant F-MLV Envs and RSV DCTD Env on mCAT-1 receptor expressing cells are shown. Target cells are added to viral particles captured by anti-GFP (MLV Env) antibody (black bars), no antibody (dark grey bars) or untreated (light grey bars). (C) Infectivity of viral particles pseudotyped with the indicated mutant F-MLV Env and RSV DCTD Env on TVA receptor expressing cells are shown. Data shown are an average of 3 experiment. Error bars indicate the SD in the experiments.

Figure 5-3: Infectious viral particle production is not affected by the presence of the leucine frameshift mutant F-MLV Envs



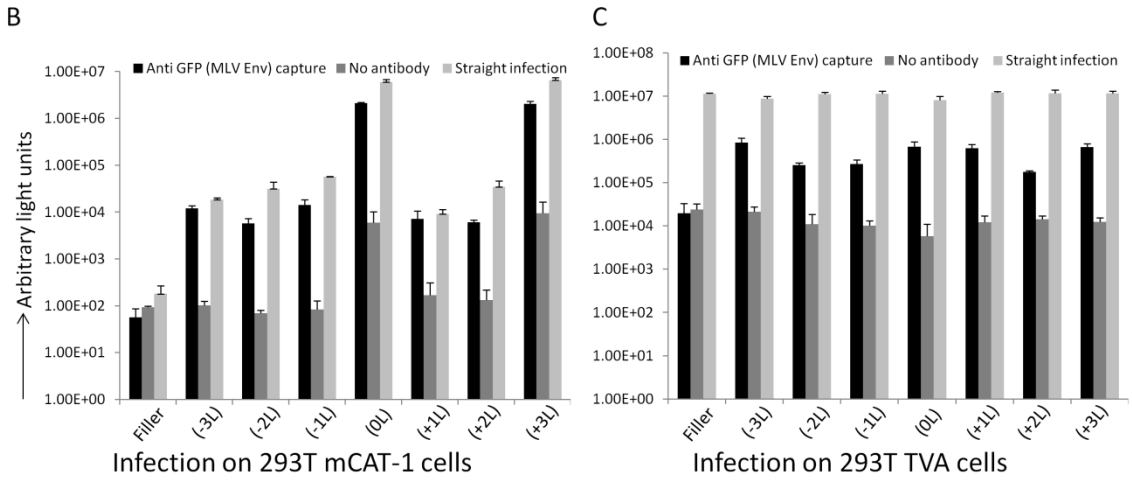


Figure 5-4 **Addition/deletion of Leucines in MSD of F-MLV glycoprotein affects fusogenecity of Env when the CTD is present**

A)Fusogenecity of the indicated F-MLV FL and ΔR Env mutants, relative to ΔR Env is shown as an average of three independent experiments. Bars indicate the SD within the experiments. (B) Infectivity of ΔR Envs with the indicated MSD mutations, relative to WT Env infectivity is shown. (C) Infectivity of Δ25 Envs with the indicated mutations in the MSD, relative to WT Env infectivity is shown.

Figure 5-4: Addition/deletion of Leucines in MSD of F-MLV glycoprotein affects fusogenecity of Env when the CTD is present

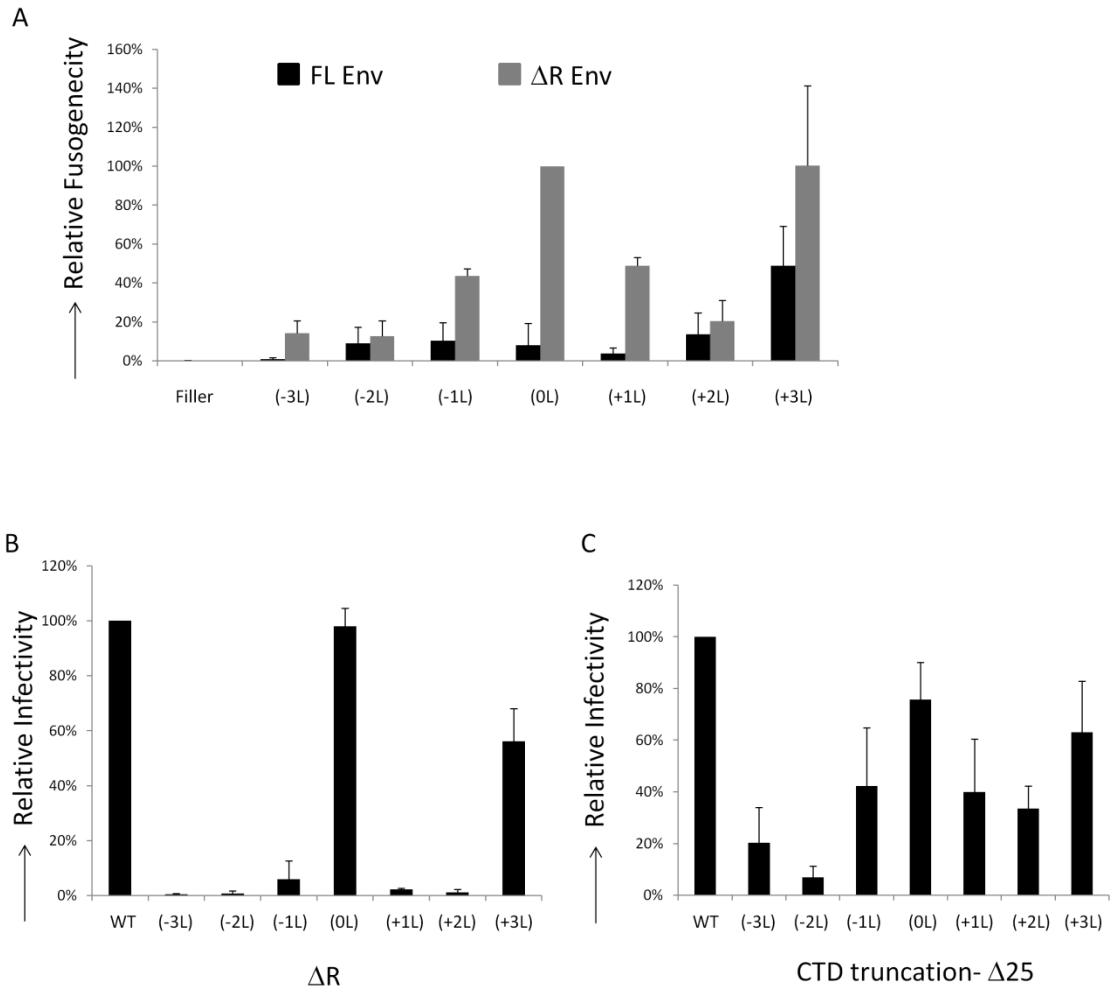


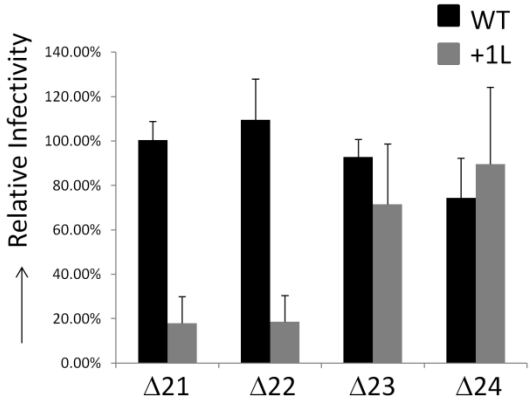
Figure 5-5: **Sequences in CTD contributing to infectivity phenotype with 0L and +1L Env**

(A) Truncations of CTD and their sequences in the C-terminus are shown. Infectivity of the various truncation mutants with WT and +1L Env are shown. Data shown are the average of at least 4 experiments. Error bars indicate the SD in the experiments. (B) Double alanine mutations in the context of WT and +1L Δ21 Envs are shown. Relative infectivity of the various mutants are shown as indicated. Data shown are the average of at least 3 experiments. Error bars indicate the SD in the experiments. (C) Single alanine mutations in the context of WT and +1L Δ21 Envs are shown. Infectivity of the various mutants are shown as indicated. Data shown are the average of at least 3 experiments. Error bars indicate the SD in the experiments.

Figure 5-5: Sequences in CTD contributing to infectivity phenotype with 0L and +1L Env

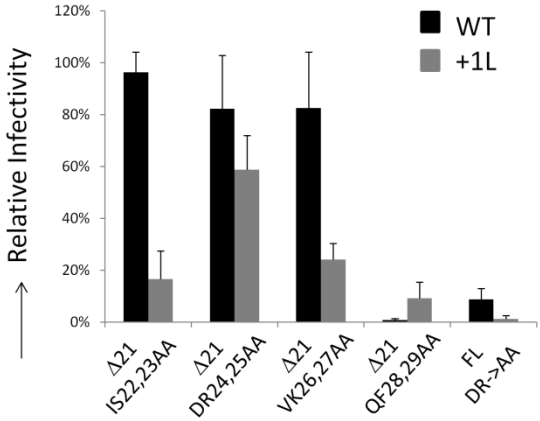
A

```
                                                    29          21                  1
FL    LISTIMGPLII LLL ILLFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPIEYEP
Δ21   ----------------------------------S*
Δ22   ----------------------------------I*
Δ23   ----------------------------------R*
Δ24   ----------------------------------D*
```



B

```
                                                  29        22
Δ21          LISTIMGPLII LLL ILLFGPCILNRLVQFVKDRIS*
22,23AA      ----------------------------------AA*
24,25AA      --------------------------------AA--*
26,27AA      ------------------------------AA----*
28,29AA      ----------------------------AA------*
FL,DR->AA    LISTIMGPLII LLL ILLFGPCILNRLVQFVKAAISVVQALVLTQQYHQLKPIEYEP
```



C

```
                                                        29        22
Δ21          LISTIMGPLIILLLILLFGPCILNRLVQFVKDRIS*
Δ21 22A      ---------------------------------A*
Δ21 23A      --------------------------------A-*
Δ21 24A      -------------------------------A--*
Δ21 25A      ------------------------------A---*
Δ21 26A      -----------------------------A----*
Δ21 27A      ----------------------------A-----*
Δ21 28A      ---------------------------A------*
Δ21 29A      --------------------------A-------*
```

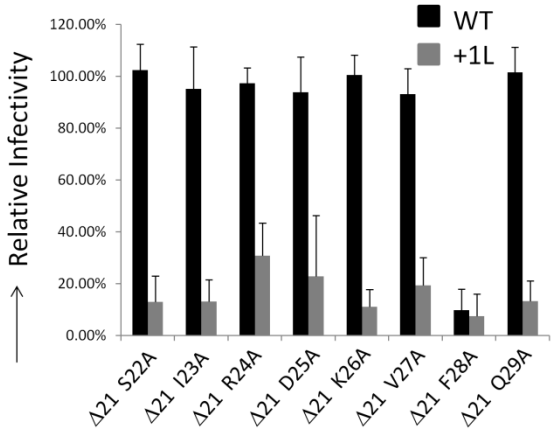


Figure 5-6: **HIV-1 Env MSD hydrophobicity does not dictate Env recruitment and infectivity modulation is independent of the CTD**

Infectivity of HIV-1, relative to WT HIV-1 Env, with the indicated HIV-1 Env and its variants are shown as an average of three independent experiments and error bars indicate the SD in the experiments.

Figure 5-6: HIV-1 Env MSD hydrophobicity does not dictate Env recruitment and infectivity modulation is independent of the CTD

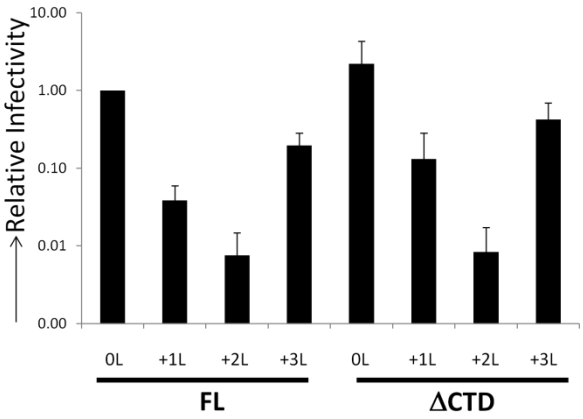


Figure 5-S1: **Infectivity with hydrophobicity change mutants**

Relative infectivity of the indicated F-MLV Env mutants are shown as an average of three experiments and the error bars indicate SD in the experiments.

Figure 5-S1: Infectivity with hydrophobicity change mutants

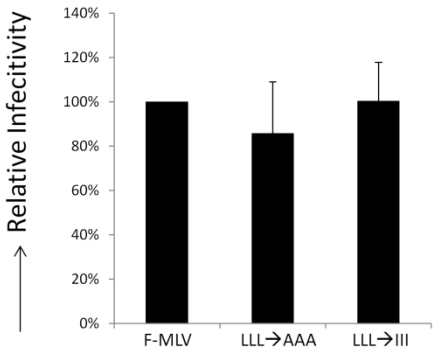


Figure 5-S2: **Infectivity with +4L and +5L mutants**

Relative infectivity of the indicated F-MLV Env mutants are shown as an average of three experiments and the error bars indicate SD in the experiments.

Figure 5-S2: Infectivity with +4L and +5L mutants

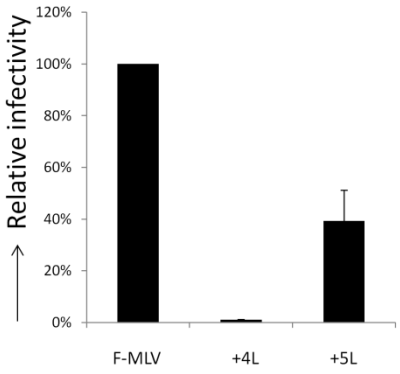
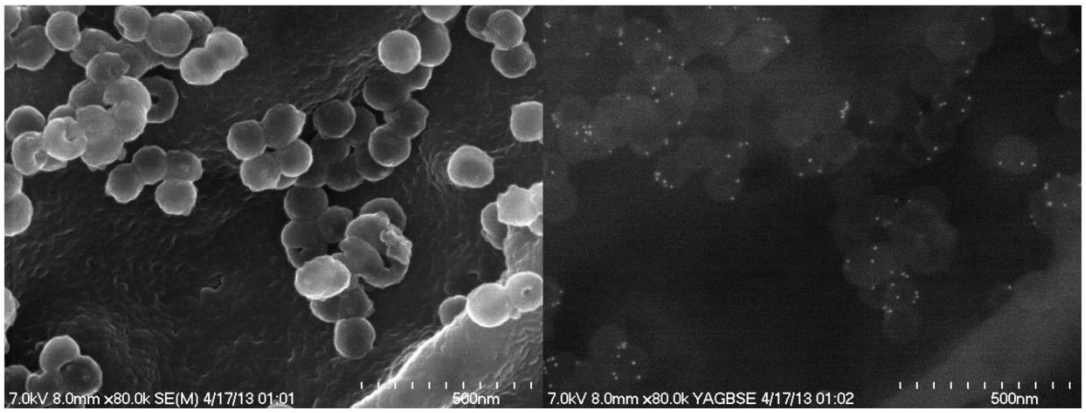


Figure 5-S3: **+1L MLV Env is specifically recruited to viral budding sites**

293T mCAT-1 cells were transfected with plasmids expressing the HIV Gag PTAP→AAAA and YFP-tagged +1L MLV Env. The cells were stained with a mouse anti- GFP monoclonal antibody and a gold-labelled anti mouse secondary antibody. Scanning electron micrographs were obtained for budding virions and back scatter detection was employed for labeled Env. Scale bars, 500 nm

Figure 5-S3: +1L MLV Env is specifically recruited to viral budding sites



VI. Summary and Discussion

Retroviruses are able to form infectious pseudotyped particles with a variety of viral fusion glycoproteins and this Pseudotyping compatibility does not follow family lines (121). However, pseudotyping of vector particles allows the targeting of specific cell types (12, 65, 95, 134, 181, 213), and this functionality has sparked a lot of interest in pseudotyped vectors. As previously mentioned, lentiviral vectors can infect dividing and non-dividing cells, leading to long-term stable expression of a transgene in the target cell. A combination thereof that possesses lentiviral vector properties and Env that targets it to different tissues would be tremendously helpful. The assembly of retroviruses has been characterized extensively. But, the mechanisms of Env acquisition have not been adequately elucidated. My work has largely shown Vpu as a modulator of specific glycoprotein intake into viral particles, and the role of a helical conformation of the Env MSD in Env fusogenicity. Also, CTD mutants of the gammaretroviral Env provide important clues about the structure of the CTD that is best suited for a native particle uptake versus a non-native particle uptake.

Previous work has shown that Vpu prevents incorporation of glycoproteins with GaLV Env CTD from coalescing with viral particles (167). Upon an initial observation, the GaLV Env CTD did not resemble any known Vpu target. Suspecting a new mechanism of Vpu sensitivity for a target protein, mutational analysis was performed on the GaLV Env CTD, which led to the characterization of a 'Vpu sensitivity motif'. This motif was also modular, allowing Vpu insensitive proteins to become sensitive.

Remarkably, a stringent look of the human glycoproteome reveals the presence of only three proteins that bear this sensitivity motif- CD4, a known Vpu target being one of them. A slightly broader look shows that several other glycoproteins may be targeted by Vpu, by virtue of the presence of an amphipathic alpha helix structure in the CTD of the respective Envs.

The study of Vpu sensitivity on GaLV Env CTD also produced a very valuable tool, in that, a library of a Gammaretroviral Env with every position in the CTD mutated, was created. Assaying for infectivity of HIV-1 particles with this entire library allowed us to determine sequences in the gammaretroviral Env CTD that allow pseudotyping of GaLV Env with HIV-1 particles. MLV Env and GaLV Env are closely related as mentioned earlier. MLV Env is an extremely well studied Env, with a trove of information available about the various domains and the mechanism of Env fusion. Comparison of studies performed on MLV Env CTD with the current study involving GaLV Env CTD shows that infectivity data in general agree with previously published studies. Also, some mutants are infectious with HIV, but not infectious with MLV virions, while some other mutants follow the reverse trend. Further studies with such mutant Envs will provide valuable clues about mechanism of pseudotyping and mechanism of recruitment of Env to different particles.

GaLV Env is thus a model glycoprotein that is targeted by Vpu and we exploited this system to understand further about Vpu sensitivity. Retroviral glycoproteins are

trimeric in nature. This trimeric nature allows for trials of *trans* complementation of Env function. Indeed, defective HIV-1 Env and MLV Env has been shown to complement each other to form functionally-active, mixed trimers (246, 313-314). Exploiting the functional complementation of the MLV Env, we further studied Vpu sensitivity of the model Vpu target, GaLV Env CTD. While Env with similar ectodomains have been shown to heterotrimerize(41), for the first time, we were able to show that Env trimers with different CTDs could efficiently trimerize with each and form a functional Env unit. More importantly, the data indicated that Vpu targets a single CTD to enforce its effect on the protein. This is an important piece of data to reconcile with, as different glycoproteins multimerize and heteromultimerize to varying extents and with this model target, we were able to show that Vpu's effect on one CTD could affect an entire complex.

Further, studies on Vpu using the GaLV Env has given us interesting insights into the conformation of the CTD of the gammaretroviral Env itself. Recently cryoelectron-tomography of the MLV Env has shown that the TM of MLV Env is in a trimeric configuration and upon R-peptide cleavage in the Env, the TM legs are splayed. This provides a structural basis for fusion activation of the MLV Env in the virus. Our data indicates that Vpu targets a single GaLV Env CTD, but only if all the three CTDs of the Env are present, indicating that Vpu targets a single helical CTD in a multimer. This observation further supports that idea that Env CTD remains a trimer in the cell.

The studies of Vpu sensitivity with GaLV Env CTD has also allowed us to develop assays that may potentially be used in inhibitor screens against Vpu. Currently, no drugs against Vpu exist. Vpu has been shown to be important for viral pathogenesis in the pig-tailed macaque model, as an animal infected with ΔVpu virus does not progress towards AIDS (268). In the presence of Vpu, GaLV Env does not pseudotype with HIV particles. However, if Vpu is inhibited or the Vpu-GaLV Env CTD interaction is inhibited, then GaLV Env would be able to produce infectious particles with HIV and this infectivity can be assayed in a high-throughput format. Exploitation of an observed phenotype towards translational research, leading to drug identification is the real benefit of this study.

In addition to screening for inhibitors, an infectivity assay with a CTD based Vpu target and an MSD based Vpu target, provided an opportunity to compare the two modes and two types of Vpu targeting. Tetherin is targeted by Vpu through the MSD and GaLV Env is targeted through its CTD, similar to CD4. Tetherin and CD4 are the most studied Vpu target proteins, but use of different assays to study the effect of Vpu on these proteins reduces the comparisons that can be made between the two modes of Vpu action. Infectivity assays with GaLV Env or with VSV-G in the presence or absence of tetherin allows us to compare these two targets directly. While GaLV Env produces infectious particles in the absence of Vpu, Tetherin prevents the release of infectious particles in the absence of Vpu. Pairwise analysis for infectivity with GaLV Env in the presence or absence of Vpu and for VSV-G Env in the presence and absence of tetherin were performed to analyse the effect of various mutations in Vpu. Surprisingly, both the

targets required the presence of the serines and surrounding amino acids in the hinge region of the Vpu CTD, although the subcellular location, where each protein was targeted was different. Given the data, further studies on the phosphorylation status of Vpu with these mutants have to be performed to understand the differences between the two targets.

The above-mentioned studies indicate the presence of a physical factor, Vpu, which prevents the recruitment of a viral or a cellular glycoprotein to HIV budding sites. With the next study, we addressed the role of the MSD in MLV Env recruitment to viral budding sites. The CTD of MLV Env is not necessary for its specific recruitment to HIV virions (168). In the case of HIV-1 Env though, CTD truncated Env mutants are non-specifically taken up by HIV virions (196). To systematically deduce Env determinants of viral pseudotyping, we examined the hydrophobic patch of amino acids in the MLV Env MSD. Changes in hydrophobicity of the MSD did not affect Env recruitment to viral particles and further did not affect specific localization of Env to budding viruses. Surprisingly, addition of three leucines to the MSD of Env partially restored infectivity with HIV-1 when compared to WT MLV Env. Such a phenotype is reminiscent of an alpha helical structure and indeed, the MSD and CTD of MLV Env is modeled to be a continuous alpha helix. Addition of leucines would distort the helix and the fusogenicity data with these mutants reflects such a phenotype. 0L ΔR and +3L ΔR Envs are fusogenic and all other mutant Envs have varying levels of fusogenicity. Cell-to-cell fusion capabilities of these Envs correlates with the infectivity output with these Envs.

Truncation of most of the CTD allows partial regain of infectivity and specific sequences within the Env CTD regulate this phenotype.

MLV is an Env whose CTD is proteolytically cleaved prior to fusogenic activation. A closer look of the HIV Env, which is not proteolytically cleaved during fusion activation, revealed a similar phenotyped. Addition of leucines in a stepwise fashion decreased infectivity and addition of three leucines partially restored infectivity to HIV Env. However, a similar pattern was noted with the ΔCTD Env, indicating that the CTD of HIV-1 Env does not regulate components of the Env MSD. This study hence showed the dependence of Env fusogenicity on helical configuration of the Env MSD. Through a variety of techniques and methods, we have also shown that the hydrophobicity of the MSD of Env does not dictate Env recruitment to virions.

VII. Future Directions

The body of work described in this dissertation provides a basis for further studies in several ways. With the creation of a library of gammaretroviral Env CTD mutants, we are in possession of a unique tool to understand several aspects of the Env CTD. For example, R-peptide cleavage has been reported to be crucial for fusion regulation of the MLV Env; But a comprehensive analysis of the Env CTD has not been performed to understand Env fusogenicity. Assays with the panel of mutants would allow us to genetically tease different sequences required for a particular phenotype attributed to the Env CTD.

The GaLV Env CTD provides an unique opportunity to study Vpu mediated effects on a target protein, where the CTD is required for modulation. Potentially, we could use GaLV Env as a stand-in model protein fro CD4 to completely understand the interaction of Vpu with CD4. Further, the Vpu sensitivity motif may also provide information about other host cellular proteins targeted by Vpu. The use of a trimeric target protein has helped us understand the action of Vpu on a multimeric target and this study may potentially be extended to other monomeric or multimeric proteins that Vpu targets.

The studies on the MSD of Env also provide insights into the fusogenic ability of Env. A comprehensive study of fusion maybe performed to understand this phenomenon completely. The differences in Envs that are regulated for fusion through proteolytic

cleavage of their CTD may be differentiated from those that are not regulated by proteolysis of Env CTD. Further work with the MSD mutants may involve understanding of the specificity of recruitment of Env to viral particles.

Progress with retroviral vectors has to match progress with understanding of how each component in the vector functions. Mechanisms of pseudotyping would provide an understanding of Env acquisition with both the native and non-native viral glycoproteins. Such understanding could then lead to creation of vectors that can be targeted to different tissues within the body with a high degree of specificity. With these hopes for the future, this dissertation is thus concluded.

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VITA

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