Cell, Vol. 93, 685–692, May 29, 1998, Copyright ©1998 by Cell Press

HIV-1 Auxiliary Proteins: Making Connections in a Dying Cell

Review

Bryan R. Cullen
Howard Hughes Medical Institute
and Department of Genetics
Duke University Medical Center
Durham, North Carolina 27710

Shortly after the first molecular clones of human immunodeficiency virus type 1 (HIV-1) were obtained, it became apparent that the genomic organization of this human retroviral pathogen was significantly more complex than seen in the oncogenic murine and avian viruses that had previously provided the focus of retroviral research. While the efficient replication of these animal retroviruses is dependent only on the biological activity of the prototypic *gag*, *pol*, and *env* gene products, HIV-1 was found to express six additional small proteins whose purpose was initially entirely unclear. It soon emerged that two of these novel proteins, termed Tat and Rev, were essential for HIV-1 replication while a third, termed Vif, was critical in many culture settings, including in primary human lymphocytes. The final three auxiliary proteins, termed Nef, Vpr, and Vpu, proved to have quite modest phenotypes in most cell culture assays. However, regardless of their in vitro phenotype, it seemed certain that all of these novel viral proteins would contribute to efficient HIV-1 replication, and hence pathogenesis, in vivo. The HIV-1 auxiliary proteins therefore rapidly became the subject of intense scientific analysis. This process has now begun to yield insights into not only the molecular mechanisms underlying the HIV-1 replication cycle but also, and more generally, into fundamental aspects of the molecular biology of eukaryotic cells.

Tat: Transcriptional Activation via an RNA Target

The Tat protein is a potent transcriptional activator of the HIV-1 long terminal repeat (LTR) promoter element and is essential for viral replication in almost all culture settings (reviewed by Cullen, 1995). The most surprising, and still unique, aspect of Tat function is that this protein acts via an RNA structure, termed TAR, located immediately 3′ to the LTR transcription start site. The TAR element forms a 59-nucleotide RNA stem-loop structure that presents two critical sequence elements, i.e., a terminal hexanucleotide loop and a three-nucleotide U-rich bulge located four nucleotides 5′ to the loop. In vitro analysis demonstrated that Tat can directly bind to the U-rich bulge present in TAR. However, this interaction is inefficient and is also independent of the TAR loop, even though both the bulge and the loop of TAR are critical for Tat function in vivo.

Mutational analysis of the short, ~86 aa Tat protein revealed the presence of two functional domains. These are a cofactor-binding domain, extending from near the amino terminus to residue 48, and an arginine-rich RNA-binding motif (ARM), extending from residue 49 to 58, that also acts as a nuclear localization signal (NLS) (reviewed by Cullen, 1995). Genetic analysis of Tat function demonstrated that cofactor binding was critical not only for activation of the HIV-1 LTR but also for TAR binding in vivo. Two observations are relevant. (1) While full-length Tat is able to target a heterologous protein to TAR RNA in vivo, this activity is blocked by mutation not only of the ARM but also of the cofactor-binding domain. (2) Tat can activate transcription effectively when targeted to the HIV-1 LTR by fusion to a heterologous RNA-binding domain. In this context, the ARM is dispensable for Tat function while the cofactor-binding domain remains essential.

The finding that Tat could activate the HIV-1 LTR effectively when targeted to a heterologous RNA sequence substituted in place of TAR also provided two other insights into Tat function. First, this result clearly demonstrated that the sole role of TAR was to recruit Tat, and any associated cellular proteins, to the HIV-1 LTR promoter. Second, the observation that Tat is unable to activate transcription from the wild-type HIV-1 LTR in mouse cells, yet is fully active when targeted to the LTR via an inserted heterologous RNA target, demonstrated that the cellular factors required for activation of the HIV-1 LTR promoter are available to bind Tat in mouse cells but recruitment to TAR is defective (Alonso et al., 1992). Overall, these studies therefore suggested that the role of Tat was to recruit a cellular cofactor(s) to TAR in a process that involved Tat binding to the TAR bulge and cofactor binding to the TAR loop. This recruitment then resulted in activated transcription from the HIV-1 LTR. In mouse cells, the equivalent cofactor was present and could still bind Tat but, for unknown reasons, the resultant Tat–cofactor complex was unable to recognize TAR. Evidence that this cofactor was likely to be a single protein came from the finding that introduction of human chromosome 12 into mouse cells was able to rescue Tat function partially (Alonso et al., 1992).

Research into the mechanism used by Tat to activate the HIV-1 LTR gave the surprising result that the level of transcription initiation from the LTR was essentially equivalent in the presence or absence of Tat. However, in the absence of Tat, these initiated transcripts almost all terminated prematurely, within ~200 nucleotides of the transcription start site (Kao et al., 1987). In contrast, in the presence of Tat, elongation was found to be efficient, thus suggesting that Tat acted to promote the processivity of initiated RNA polymerase II (Pol II) molecules. Although the regulation of Pol II processivity remains incompletely understood, this activity is known to be modulated by the phosphorylation state of the C-terminal domain (CTD) of Pol II, which consists of tandem reiterations of a serine-rich 7 aa repeat. In particular, the CTD is not phosphorylated when Pol II is bound at the promoter but hyperphosphorylated on efficiently elongating Pol II molecules. It is therefore believed that CTD phosphorylation is critical for Pol II release from the promoter and for processive transcription. Could Tat be acting by enhancing the phosphorylation state of the Pol II CTD? Two findings suggested that this might indeed be the case. First, removal of the CTD was found to block the ability of Pol II to respond to Tat, and second, Tat function was found to be effectively inhibited

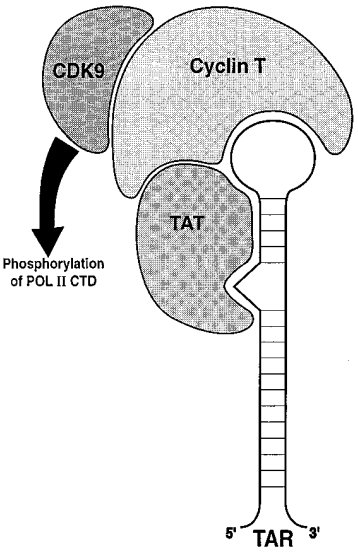

Figure 1. Model for the Assembly of the Tat–cyclin T–CDK9 Heterotrimer onto the HIV-1 TAR Element

by DRB, a nucleoside analog that functions both as a selective kinase inhibitor and as an inhibitor of transcription elongation by Pol II (Yang et al., 1996).

A critical finding in support of the hypothesis that Tat acted at the level of CTD phosphorylation was the observation that immunoprecipitation of Tat resulted in the specific coimmunoprecipitation of a CTD kinase of ~42 kDa (Yang et al., 1996). Research into factors involved in transcription elongation in Drosophila had meanwhile identified an essential multicomponent factor, termed positive transcription elongation factor b (P-TEFb), that included a novel CTD kinase with homology to cyclin-dependent kinases (CDKs) (Zhu et al., 1997). Identification of the human homolog of this protein, now called CDK9, revealed that this was also ~42 kDa in size. It was rapidly demonstrated that the kinase that associates with Tat in vivo was indeed CDK9 and that CDK9 mutants lacking kinase activity could selectively inhibit Tat function (Zhu et al., 1997).

While the identification of CDK9 as a critical mediator of Tat function represented a key step forward, the story remained incomplete. Thus, although CDK9 associated with Tat in vivo, no direct interaction between Tat and CDK9 could be demonstrated in vitro, thus making it likely that this interaction was indirect. Could the expected cyclin partner for CDK9 be the missing link? In fact, recent data from Wei et al. (1998) demonstrate that the Tat activation domain, but not mutant forms thereof, is able to interact directly with a protein termed cyclin T that, in turn, can bind specifically to CDK9. Importantly, the Tat–cyclin T complex binds to TAR with high affinity and specificity in vitro in an interaction that is dependent on the integrity of not only the bulge but also the loop of TAR. Particularly satisfying is the observation that cyclin T is encoded on human chromosome 12 and that overexpression of human cyclin T in mouse cells rescues Tat transcriptional activation via the HIV-1 TAR element. Therefore, the role of Tat appears to be to bind the cyclin T subunit of P-TEFb and to recruit P-TEFb to the HIV-1 LTR promoter, a process that requires binding of Tat to the TAR bulge and of cyclin T to the TAR loop (Figure 1). The cyclin T–associated CDK9 kinase then induces phosphorylation of the CTD of Pol II, and perhaps other pol II–associated proteins, leading to a transition from nonprocessive to processive transcription.

The mechanism of transcription activation of the HIV-1 LTR by Tat may therefore be close to resolution. Remaining questions include whether the Pol II CTD is the only target for P-TEFb and whether other P-TEFb subunits play a critical role in this process. It is also interesting to speculate as to why the HIV-1 LTR has evolved to promote the initiation of almost entirely nonprocessive transcription complexes that must then be rescued by Tat, when transcription complexes initiated by LTRs found in most other retroviruses elongate effectively without such rescue. While it is tempting to suggest that this property may somehow facilitate the establishment of HIV-1 latency, this must be viewed as little more than speculation at present.

Rev: Nuclear RNA Export Factor

The large majority of genes in higher eukaryotes exist in the form of multiple coding exons separated by noncoding, intronic regions. These genes are therefore transcribed in the form of pre-mRNAs that must be extensively spliced before the mature mRNA, encoding the relevant protein product, is ready for translation. Because these pre-mRNA intermediates would encode useless or even deleterious proteins if available to the cytoplasmic translational machinery, the eukaryotic cell has evolved mechanisms to retain incompletely spliced RNAs in the nucleus until splicing is complete. While the mechanisms that maintain this segregation remain to be fully elucidated, this is likely to involve the recognition of unused 5′ splice sites by splicing commitment factors such as the U1 small nuclear ribonucleoprotein particle and members of the serine-arginine-rich class of splicing factors.

While the nuclear retention of incompletely spliced RNAs is undoubtedly benefical to the host cell, this presents retroviruses in general, and complex retroviruses in particular, with a serious problem (reviewed by Cullen, 1995). Because HIV-1 contains only the single LTR promoter element, it encodes only a single, genome-length primary transcript. Yet expression of the nine open reading frames encoded by HIV-1 requires that this single transcript be expressed in the cytoplasm as an unspliced mRNA that serves both as the viral genome and as the mRNA for Gag and Pol—as one of five singly spliced mRNAs, encoding Vif, Vpr, Vpu, and Env and lastly as one of the 16 multiply or completely spliced mRNAs encoding Tat, Rev, and Nef. Because splicing of HIV-1 RNA transcripts is performed entirely by cellular proteins, the generation of singly and multiply spliced viral mRNAs requires that the viral genome encode 5′ and 3′ splice sites that can be recognized by cellular splicing factors. However, the presence of such splice sites leads to the nuclear retention of incompletely spliced viral transcripts and hence precludes expression of proteins encoded by the unspliced (Gag, Pol) and singly spliced (Env, Vif, Vpr, Vpu) viral mRNAs. Note

however that HIV-1 transcripts that do not retain unutilized 5′ splice sites, i.e. the fully spliced mRNAs encoding Tat, Rev and Nef, are not subject to nuclear retention.

To overcome this problem, HIV-1 has adopted two strategies. The first is that the splice sites present in the HIV-1 genome are designed to be inefficient. While this is not, in and of itself, sufficient to permit the nuclear export of incompletely spliced HIV-1 transcripts, it does permit a pool of such RNAs to accumulate in the nucleus. Second, HIV-1 has evolved a sequence-specific nuclear RNA export factor, termed Rev, that is able to induce the efficient nuclear export, and hence expression, of the various incompletely spliced viral transcripts (Malim et al., 1989). Because Rev, Tat, and Nef are encoded by fully spliced HIV-1 mRNAs, these gene products are expressed shortly after infection of cells and are therefore referred to as early gene products. In contrast, the Gag, Pol, Env, Vif, Vpr, and Vpu proteins are all dependent on Rev for the nucleocytoplasmic transport of their cognate mRNAs and are therefore expressed with delayed kinetics. These six proteins are therefore referred to as late HIV-1 proteins.

The ~116 aa Rev protein contains an arginine-rich stretch located toward the amino terminus that serves as both an ARM and as an NLS (reviewed by Cullen, 1995). This is, in turn, closely flanked on both sides by residues that mediate Rev multimerization. Unlike the Tat ARM, the ARM present in Rev is fully able to mediate binding to its cognate RNA target site without the assistance of a cellular cofactor. The RNA target for Rev is a 234-nucleotide RNA stem-loop structure called the Rev response element (RRE) encoded within the HIV-1 *env* gene (Malim et al., 1989). Rev binds, most probably as a monomer, to an RNA bulge within the RRE. This initial binding event then serves to nucleate the recruitment of additional Rev monomers to the RRE in a multimerization process that requires both protein–protein and protein–RNA interactions and that is critical for Rev-mediated nuclear RNA export. The second functional domain in Rev is an ~10 aa leucine-rich sequence, located between residues 75 and 84, that functions as a nuclear export signal (NES) both in Rev and when attached to other substrate proteins (Fischer et al., 1995). Because Rev contains both an NLS and an NES, it rapidly shuttles back and forth between the nucleus and the cytoplasm of expressing cells.

Initial efforts to identify a protein that mediated the biological activity of the Rev NES, using the yeast two-hybrid assay, demonstrated a highly specific interaction between the Rev NES and certain components of the nuclear pore, termed nucleoporins. Because nuclear pores regulate all nucleocytoplasmic transport (reviewed by Mattaj and Englmeier, 1998), this result was exciting in that it suggested that Rev might act directly to recruit RRE-containing RNAs to the nuclear pore and hence to the cytoplasm. More recently, it has become apparent that this interaction, while real, is in fact bridged by a protein, called Crm1, that is highly conserved between yeast and humans (Fornerod et al., 1997; Neville et al., 1997; Stade et al., 1997). Crm1 is a member of a group of related proteins, of which the prototype is Importin β (Imp β), that mediate the regulated nuclear import and export of proteins and RNAs and that are known to

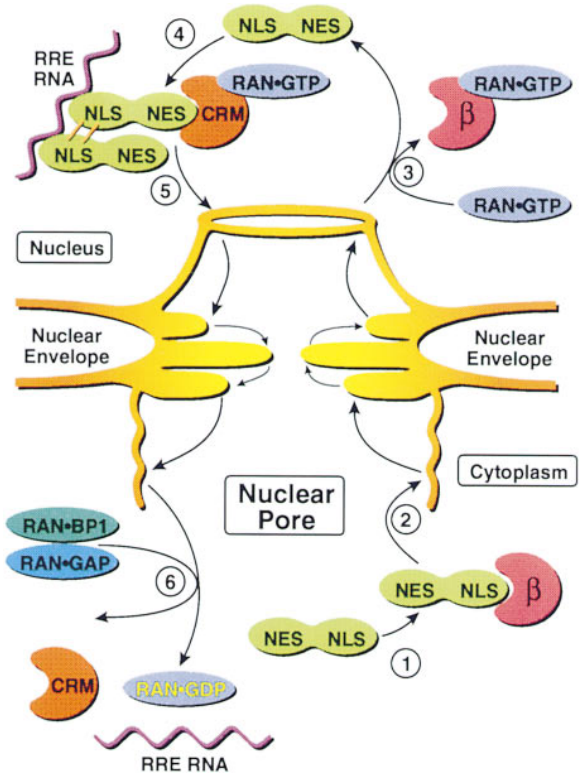

Figure 2. Overview of the Steps Involved in Rev Nucleocytoplasmic Shuttling

Numbers are referred to in the text.

interact directly with nucleoporins (Mattaj and Englmeier, 1998). Binding of import factors, such as Imp β, or export factors, such as Crm1, to cargo proteins is in turn regulated by the G protein Ran, which is found in the GTP-bound form in the nucleus and in the GDP-bound form in the cytoplasm. The proposed steps in the Rev nucleocytoplasmic transport cycle are numbered in Figure 2.

After synthesis, the Rev protein directly interacts with Imp β via its NLS (1) (Henderson and Percipalle, 1997). This is actually unusual, in that most basic NLS proteins interact with an adaptor protein, termed Importin α (Imp α), that in turn binds to Imp β. In either case, Imp β, together with its protein cargo, is then recruited to the nuclear pore by a direct Imp β–nucleoporin interaction (2). The process by which translocation into the nucleoplasm then occurs is unclear; however, Imp-mediated nuclear import is known to require energy and may involve the ordered, sequential interaction of Imp β with specific nucleoporins. Once the Imp β–Rev NLS complex reaches the nucleus, where Ran–GTP is present at high levels, the direct interaction of Imp β with Ran–GTP results in release of the Rev cargo (3).

The next step in the Rev pathway involves the specific assembly of multiple Rev molecules onto the RRE and recruitment of Crm1 to the Rev NES sequences thereby assembled onto the target HIV-1 transcript (4). As noted above, Crm1 is a member of the same family of nucleocytoplasmic transport factors as Imp β and shares a conserved Ran–GTP-binding domain. However, while

Ran–GTP binding by import factors such as Imp β induces the nuclear release of cargo proteins, Ran–GTP binding is a prerequisite for binding of cargo proteins by nuclear export factors such as Crm1. The resultant ribonucleoprotein complex, consisting of the RRE RNA and multiple Rev, Crm 1 and Ran–GTP molecules, is then recruited to the nuclear pore via the direct interaction of Crm1 with nucleoporins referred to above (5). The translocation through the nuclear pore that follows is again not understood, but can be viewed as the mirror image of the import translocation mediated by Imp β. Once the HIV-1 RNA cargo reaches the cytoplasm, the Ran GTPase activating protein (RanGAP), acting in concert with Ran Binding Protein 1 (RanBP1), induces the hydrolysis of Crm1 bound Ran-GTP to Ran-GDP (6). This releases both Ran and Crm1 from the Rev NES. It is unclear how Rev is released from the HIV-1 RNA, although this could reflect competition from cytoplasmic Imp β for binding to the Rev basic domain. At this point, the cargo HIV-1 RNA is available for translation of the encoded late viral protein while Rev is free to once more return to the nucleus.

Nef: Numerous Effector Functions

The third early protein encoded by HIV-1, termed Nef, is not only the largest auxiliary protein, at ~206 aa in length, but is also expressed in far higher levels than Tat and Rev. Nevertheless, Nef was initially found to exert a quite modest effect on the rate of HIV-1 replication in culture, with some early reports even suggesting that Nef was a **ne**gative **f**actor. However, the subsequent demonstration that inactivation of the *nef* gene in a pathogenic clone of simian immunodeficiency virus (SIV) caused a dramatic drop in both viral titer and pathogenic potential in infected macaques, revealed that Nef was critically important in vivo (Kestler et al., 1991). More recent research has demonstrated that Nef exerts at least three distinct activities in infected cells.

Down-Regulation of Cell Surface CD4

The first clear activity to be assigned to Nef was down-regulation of cell surface CD4 expression (Garcia and Miller, 1991). CD4 is the primary receptor for HIV-1 and down-regulation would therefore be predicted to reduce the formation of complexes between CD4 and newly synthesized HIV-1 Envelope protein on the infected cell surface and also to facilitate the release of HIV-1 virions (Benson et al., 1993). The Nef protein is posttranslationally modified by myristoylation of its amino terminus, and this modification targets Nef to the inner surface of the plasma membrane and to the trans-Golgi network (TGN). It is believed that Nef binds the cytoplasmic tail of CD4 directly, although this remains to be fully proven. Downregulation of cell surface CD4 is a specific event requiring a cluster of leucine and isoleucine residues in the CD4 cytoplasmic tail and results from the targeting of CD4 into clathrin coated pits (CCPs) followed by internalization and transport to lysosomes, where CD4 is degraded (Aiken et al., 1994). Direct fusion of Nef to the extracellular and transmembrane domains of CD4 recapitulates the phenotype seen when Nef is expressed in *trans*, i.e. the CD4-Nef fusion protein is also internalized via CCPs and degraded. This observation raised the possibility that Nef was acting as a connector between CD4 and the cellular endocytic machinery.

Internalization of cell surface receptors via CCPs requires the action of adaptor protein (AP) complexes that bind to both clathrin and either directly to the receptor itself or indirectly, via a connector protein. AP complexes exist in two major varieties termed AP-1, found in the TGN, and AP-2, found at the plasma membrane. While both AP complexes are heterotetramers of similar size and structure, there are no subunits in common. Analysis of the ability of Nef to interact with the various subunits of AP-1 and AP-2 has recently demonstrated that Nef can directly interact with both the μ1 component of AP-1 and the related (40% identity) μ2 component of AP-2 (Le Gall et al., 1998; Piguet et al., 1998). As AP-2 is the adaptor present at the plasma membrane, and as internalization from the plasma membrane is the major mechanism for CD4 down-regulation, it appears probable that CD4 internalization primarily results from the recruitment of CD4 to CCPs due to the direct interaction of Nef with both CD4 and the μ2 subunit of AP-2.

Down-Regulation of Cell Surface MHC I

In addition to CD4, Nef also induces the specific down-regulation of cell-surface MHC I receptors, albeit with somewhat lower efficiency (Le Gall et al., 1998). MHC I down-regulation results from both the internalization of cell-surface MHC I receptors and from the sorting of MHC I molecules from the TGN into AP-1-containing clathrin-coated vesicles. This down-regulation requires specific sequences in the MHC I cytoplasmic tail including a key tyrosine residue (Greenberg et al., 1998; Le Gall et al., 1998). No leucine motif, similar to that required for CD4 down-regulation, exists in MHC I.

Because MHC I is required to present viral peptide epitopes to cytotoxic T lymphocytes (CTL), down-regulation of cell surface MHC I could inhibit the CTL-mediated lysis of HIV-1-infected cells, and this has indeed been recently demonstrated (Collins et al., 1998). Although this result might suggest that MHC I down-regulation is the major contributor to the in vivo Nef phenotype, this may not be the case as a clear positive effect of Nef on viral replication in vivo is seen in SIV-infected macaques by two weeks after infection (Kestler et al., 1991), i.e., before an effective CTL response can be mounted by the infected animal. The fact that a readily detectable anti-HIV-1 CTL response is observed in most infected individuals (reviewed by McMichael, 1998 [this issue of *Cell*]) further demonstrates that the Nef-mediated inhibition of antigen presentation via MHC I is clearly incomplete.

Analysis of the Nef protein demonstrates that CD4 and MHC I down-regulation can be, at least in part, mutationally segregated (Greenberg et al., 1998). However, as the cytoplasmic tails of CD4 and MHC I display no sequence homology, this segregation could simply reflect the selective disruption of the MHC I and CD4 targeting functions of Nef. At present, the simplest interpretation of the available data is that Nef connects both CD4 and MHC I to the intracellular protein sorting machinery by binding to CD4 or MHC I on the one hand and either AP-1 or AP-2 on the other. Regardless of whether AP-1 or AP-2 is recruited, which may largely depend on whether recruitment occurs at the TGN or

at the plasma membrane, these target proteins are then sorted into clathrin-coated vesicles that deliver them for degradation in lysosomes.

Enhanced Virion Infectivity

In addition to a possible enhancement in virion infectivity due to CD4 down-regulation, Nef also enhances virion infectivity by a second, entirely CD4-independent mechanism (Spina et al., 1994). This enhancement is conferred by Nef during the process of virion assembly and cannot be complemented by expression of Nef in target cells. While the effect of Nef on virion infectivity is quite modest in most culture settings, this effect may increase to 10-fold or more when primary T cells are infected with HIV-1 while quiescent and then subsequently activated or if certain highly susceptible target cells, such as $CD4^+$-HeLa cells, are used.

While HIV-1 virions produced in the presence and absence of Nef do not differ in terms of cell-free reverse transcriptase activity or in terms of their ability to bind to and enter target cells, they are less able to complete proviral DNA synthesis. While Nef does not cause any major change in the morphology or composition of virions, two differences have been reported. First, Nef itself is packaged into virions at low efficiency ($\leq$10 molecules per virion) and even undergoes specific processing by the HIV-1 protease. While this finding may be important, it could also simply reflect a low level of nonspecific packaging of the membrane-associated Nef protein into virions during viral budding from the cell membrane. It has also been reported that serine phosphorylation of the matrix component of HIV-1 Gag is modified in Nef-expressing cells. Nef has previously been reported to associate specifically with a serine kinase related to p21-activated kinase (PAK), and it is therefore possible that Nef may recruit a PAK-like kinase to sites of virion assembly (Swingler et al., 1997). While a Nef-induced change in the phosphorylation state of matrix could certainly affect virion infectivity, this hypothesis remains to be proven.

Effects on Cellular Signal Transduction and Activation

While there have been number of reports documenting effects of Nef on signal transduction pathways and on the activation state of both lymphoid and nonlymphoid cells, no clear consensus exists as to cellular targets or even as to whether the effect of Nef is positive or negative. Clearly, the possibility exists that these reported effects are entirely secondary to the more fully documented properties of Nef listed above. For example, Nef-induced degradation of CD4 is known to result in the release of the normally CD4-bound tyrosine kinase lck, and this could have a marked effect on signaling via, for example, the TCR/CD3 complex. In addition, Nef is believed to recruit a serine/threonine kinase related to PAK to the plasma membrane and also contains an SH3-binding motif that could interact with membrane-bound tyrosine kinases or other signaling molecules. By juxtaposing enzymes and proteins that are not normally in contact, Nef may either inadvertently or intentionally modulate the activation state of the cell. At present, the assays used to measure these effects, and the range of Nef mutants that have been analyzed, do not permit any clear statement to be made as to whether the reported effect of Nef on cellular activation represents an independent phenotype or whether this is likely to be important in vivo.

In conclusion, it is clear that Nef exhibits a number of distinct activities that undoubtedly each contribute to the marked increase in viral replication and pathogenicity displayed by Nef expressing HIV-1 and SIV in vivo. Definition of the precise contribution of each activity to the overall in vivo phenotype will require a far more complete understanding of the domain organization of Nef than currently exists. Once this is achieved, it may then be possible to design mutations that selectively inactivate specific Nef functional domains and then assess their effect on viral replication and pathogenesis in vivo.

Vif: Virion Infectivity Factor

The Vif protein is encoded by a singly spliced mRNA whose expression is Rev dependent, and Vif is therefore a late HIV-1 gene product. Vif is $\sim$192 aa in length and is expressed at high levels in the cytoplasm of infected cells. A substantial fraction of Vif is membrane associated and colocalizes with the HIV-1 Gag protein, a property that is likely to be important for its biological activity.

The only known biological activity of Vif is to enhance the infectivity of HIV-1 virions produced in primary T cells, and in "nonpermissive" cell lines such as H9, by $\sim$100-fold (Gabuzda et al., 1992). In contrast, virions produced in "permissive" cell lines such as CEM-SS are equivalently infectious whether Vif is present or not. It remains unclear whether this phenomenon reflects the presence of an inhibitor of viral replication in nonpermissive cells that is inactivated by Vif or, alternately, the existence of a factor in permissive cells that substitutes for Vif. The nonpermissive state appears most physiologically relevant, both because this is the phenotype of primary cells in culture and because *vif*$^-$ SIV replicates extremely poorly in vivo.

Vif-enhanced infectivity is conferred in the virus-producing cell yet only manifests itself in the target cell. *vif*$^-$ proviruses can therefore be complemented in *trans* in virus-producing cells but not in target cells. Analysis of HIV-1 virions produced by nonpermissive cells in the presence and absence of Vif has failed to document any quantitative or qualitative difference in the incorporation or processing of viral structural proteins, although there are reports suggesting that *vif*$^-$ viruses are more likely to exhibit an aberrant morphology. One clear difference is that between 10 and 100 molecules of Vif are packaged into virions produced in the presence of Vif. While this incorporation could certainly be important for the Vif phenotype, it remains uncertain whether this incorporation is specific as murine leukemia virus, which lacks a protein equivalent to Vif, is able to package Vif with comparable efficiency (Camaur and Trono, 1996).

There is general agreement that defective *vif*$^-$ virions can bind to, and penetrate into, target cells effectively and are also able to initiate reverse transcription (Simon and Malim, 1996). There is also general agreement that reverse transcription fails to go to completion, although there is some controversy as to the precise stage at which failure occurs. In any event, although the preintegration complex (PIC) reaches the nucleus, little or no

full-length provirus is produced and the partial reverse transcripts that are synthesized are rapidly degraded. It therefore appears that Vif, acting in the virus-producing cells, somehow modifies HIV-1 virions in a way that confers enhanced postpenetration stability and function on PICs in target cells.

Although no cellular target for Vif is as yet known, recent data demonstrate that such a target must exist (Simon et al., 1998). Specifically, while HIV-1 Vif can complement the infectivity of *vif*$^-$ HIV-1 and SIV_{AGM} virions produced in nonpermissive human cells, SIV_{AGM} Vif is inactive. In contrast, SIV_{AGM} Vif can complement *vif*$^-$ HIV-1 and SIV_{AGM} produced in simian cells, while HIV-1 Vif is inactive. These data not only demonstrate that Vif function is dependent on the presence of a cellular cofactor but also reveal that different immunodeficiency virus Vif proteins have evolved a considerable degree of primate species specificity. This result is of interest given current efforts to develop simian and murine models that can support a high-titer, pathogenic infection by HIV-1.

Vpr: Nuclear Import and G2 Arrest

The Vpr protein is a late HIV-1 gene product of ~96 aa in length that is packaged into the virion nucleocapsid in molar amounts equivalent to those of the Gag protein. Vpr may therefore be viewed as a virion structural protein. Packaging into virions is mediated by the p6 protein, located at the very carboxy terminus of the p55 Gag precursor, and also requires specific sequences located toward the center of Vpr (Paxton et al., 1993).

Analysis of the effect of Vpr on HIV-1 replication demonstrated a modest positive effect in T cell lines and primary T cells but a far more marked effect on HIV-1 replication in growth-arrested cells, such as primary macrophages. This effect is thought to reflect an important role for Vpr in mediating the nuclear import of HIV-1 PICs into the nucleus of, particularly, nondividing cells (Heinzinger et al., 1994). Nuclear import of HIV-1 PICs is an active process that is believed to require an array of NLS sequences provided by the matrix protein, by integrase, and also by Vpr. The Vpr NLS, which extends over essentially the entire amino-terminal 70 aa of Vpr, is a nonconventional NLS, i.e., distinct from the basic NLS prototype. Interestingly, fusion of a carrier protein, such as β-gal, to the Vpr NLS induces not only nuclear import, but also a marked accumulation of the fusion protein at nuclear pores (Vodicka et al., 1998). A search for potential cellular target proteins for Vpr has demonstrated that Vpr can directly interact with a subset of nucleoporins, thus potentially explaining this localization. In addition, Vpr can also bind to Imp α, the adaptor protein that normally mediates the interaction of basic NLS sequences with the Imp β nuclear import factor (Mattaj and Englmeier, 1998). However, the binding sites for Vpr and basic NLSs on Imp α are distinct and this interaction is not believed to recruit Vpr to Imp β (Popov et al., 1998). While the reason for the interaction of Vpr with Imp α is therefore uncertain, it has been proposed that it could enhance the affinity of Imp α for basic NLSs present on HIV-1 PICs, and thereby enhance PIC import into the nucleus (Popov et al., 1998). In any case, the interaction of Vpr with nucleoporins and Imp α appears likely to be critical for its role in HIV-1 PIC nuclear import and, hence, in enhancing HIV-1 replication in macrophages and other nondividing cells.

In addition to its role in nuclear import, Vpr has a second, distinct activity in expressing cells, induction of arrest in the G2 phase of the cell cycle, that maps to an ~26 aa carboxy-terminal basic domain. Cells expressing Vpr contain very low levels of $p34^{cdc2}$–cyclin B kinase activity, although both proteins are expressed (Re et al., 1995). The activity of the $p34^{cdc2}$–cyclin B kinase is critical for the transition from G2 to mitosis and requires the removal, by the phosphatase cdc25C, of phosphate residues on $p34^{cdc2}$ that inhibit kinase function. However, in Vpr-expressing cells, cdc25C is in an inactive form, thus suggesting that the target for Vpr function is likely to be an upstream regulator of the G2 to M transition.

An interesting rationale for the Vpr-mediated arrest of cells in G2 is provided by the recent observation that the HIV-1 LTR promoter is more active in G2-arrested cells (Goh et al., 1998). Arrest of cells in G2 by either Vpr or by overexpression of a dominant negative form of $p34^{cdc2}$, was shown to activate the HIV-1 LTR promoter to an equivalent degree. Therefore, the mild stimulatory effect of Vpr on the LTR promoter, which had been noted previously by others, is likely to be entirely indirect. The importance of this enhancement is, however, unclear given that the effect of Vpr on the rate of HIV-1 replication in rapidly dividing T cells in culture, which should be highly responsive to G2 arrest, is minor (Heinzinger et al., 1994; Vodicka et al., 1998).

Vpu: Unique to HIV-1

While the other auxiliary proteins of HIV-1 are conserved in some or all of the animal lentiviruses (Tat, Rev, and Vif) or at least in all primate immunodeficiency viruses (Nef and Vpr), Vpu appears unique to HIV-1 and the closely related SIV_{cpz} isolates. Vpu is an oligomeric integral membrane protein consisting of an amino-terminal transmembrane domain and a carboxy-terminal cytoplasmic tail. Vpu serves two independent functions in the HIV-1 life cycle: enhancement of virion release from infected cells and the selective degradation of CD4 in the cell endoplasmic reticulum (ER).

Simultaneous synthesis of both HIV-1 envelope and CD4 in a single cell results in the formation of Env–CD4 complexes in the ER that are retained and eventually degraded. Vpu directly interacts with a specific target sequence in the cytoplasmic tail of CD4, distinct from that seen by Nef, to target CD4 to an ER-associated protein degradation pathway. This permits the release of Env from the ER and its subsequent incorporation into progeny virions (Willey et al., 1992).

The mechanism by which Vpu targets CD4 for proteolysis has recently been resolved (Margottin et al., 1998). Vpu, via its cytoplasmic tail, binds to CD4 molecules that have been retained in the ER and also recruits a cellular factor termed h-βTrCP to the ER membrane. The h-βTrCP protein consists of a series of WD repeat elements, which bind Vpu, and a so-called F box, which recruits a cellular targeting factor for ubiquitin-mediated

Table 1. Biological Activities of Several HIV-1 Auxilary Proteins

Auxiliary Protein	Activity	Mechanism
Tat	Transcriptional activation of HIV-1 LTR	Recruitment of cyclin T to TAR RNA
Rev	Nuclear Export of late HIV-1 mRNAs	Recruitment of Crm1 to RRE RNA
Nef	Down-regulation of cell surface	Recruitment of AP-1 or AP-2 to CD4
Nef	Down-regulation of cell surface	Recruitment of AP-1 or AP-2 to MHC I
Vpu	Degradation of CD4 in the ER	Recruitment of h-βTrCP to CD4

proteolysis termed Skp1. This recruitment targets CD4 for degradation, most probably by the proteasome, while Vpu is apparently recycled (Margottin et al., 1998).

While CD4 degradation is mediated by sequences located in the Vpu cytoplasmic tail, enhanced virion release is dependent on the hydrophobic amino-terminal transmembrane domain. Vpu appears to facilitate virion release both by promoting the budding of virions from plasma, as opposed to intracytoplasmic, membrane structures and by facilitating the release of budding virions from the membrane (Klimkait et al., 1990). Mutation of Vpu therefore results in a drop in released virus and in an accumulation of intracellular virus particles. This effect appears relatively nonspecific, in that Vpu has also been reported to facilitate the budding of unrelated retroviruses. Little is known as to the mechanism responsible for this effect, although the Vpu membrane anchor, which is responsible for this phenotype, has been proposed to form a cation-selective ion channel. In vitro analysis of the effect of Vpu on HIV-1 replication rates showed a moderate positive effect and demonstrated that most of this increase results from the virion release activity of Vpu.

HIV-1 Auxiliary Proteins as Molecular Connectors

Much remains to be discovered about the many roles of the auxiliary proteins of HIV-1 in the viral life cycle, and the list of functions delineated above could well be incomplete. Nevertheless, clear progress in defining the mechanisms of action, and cellular targets, for several key auxiliary proteins has now been made. Surprisingly, as summarized in Table 1, it appears that a similar strategy has been adopted by several different auxiliary proteins to achieve a range of distinct biological activities. In particular, it appears that a common mode of action for several of these proteins is to act as a molecular connector between two macromolecules that would otherwise be unable to interact. By this simple expedient, these small proteins are each able to recruit entire cellular metabolic pathways to the cause of enhancing the efficiency of virus replication.

References

Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E., and Trono, D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell *76*, 853–864.

Alonso, A., Derse, D., and Peterlin, B.M. (1992). Human chromosome 12 is required for optimal interactions between Tat and TAR of human immunodeficiency virus type 1 in rodent cells. J. Virol. *66*, 4617–4621.

Benson, R.E., Sanfridson, A., Ottinger, J.S., Doyle, C., and Cullen, B.R. (1993). Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. J. Exp. Med. *177*, 1561–1566.

Camaur, D., and Trono, D. (1996). Characterization of human immunodeficiency virus type 1 Vif particle incorporation. J. Virol. *70*, 6106–6111.

Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature *391*, 397–401.

Cullen, B.R. (1995). Regulation of HIV gene expression. AIDS *9*, 19–32.

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W., and Lührmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475–483.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051–1060.

Gabuzda, D.H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W.A., and Sodroski, J. (1992). Role of *vif* in replication of human immunodeficiency virus type 1 in $CD4^+$ T lymphocytes. J. Virol. *66*, 6489–6495.

Garcia, J.V., and Miller, A.D. (1991). Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. Nature *350*, 508–511.

Goh, W.C., Rogel, M.E., Kinsey, C.M., Michael, S.F., Fultz, P.N., Nowak, M.A., Hahn, B.H., and Emerman, M. (1998). HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr *in vivo*. Nature Med. *4*, 65–71.

Greenberg, M.E., Iafrate, A.J., and Skowronski, J. (1998). The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. EMBO J. *17*, 2777–2789.

Heinzinger, N.K., Bukrinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.-A., Gendelman, H.E., Ratner, L., Stevenson, M., and Emerman, M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA *91*, 7311–7315.

Henderson, B.R., and Percipalle, P. (1997). Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human Importin-β. J. Mol. Biol. *274*, 693–707.

Kao, S.-Y., Calman, A.F., Luciw, P.A., and Peterlin, B.M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by *tat* gene product. Nature *330*, 489–493.

Kestler, H.W., III, Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D., and Desrosiers, R.C. (1991). Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell *65*, 651–662.

Klimkait, T., Strebel, K., Hoggan, M.D., Martin, M.A., and Orenstein, J.M. (1990). The human immunodeficiency virus type 1-specific protein Vpu is required for efficient virus maturation and release. J. Virol. *64*, 621–629.

Le Gall, S., Erdtmann, L., Benichou, S., Berlioz-Torrent, C., Liu, L., Benarous, R., Heard, J.-M., and Schwartz, O. (1998). Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. Immunity *8*, 483–495.

Malim, M.H., Hauber, J., Le, S.-Y., Maizel, J.V., and Cullen, B.R. (1989). The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature *338*, 254–257.

Margottin, F., Bour, S.P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998). A novel human WD protein, h-βTrCP, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Mol. Cell *1*, 565–574.

Mattaj, I.W., and Englmeier, L. (1998). Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. *67*, 265–306.

McMichael, A. (1998). Cell *93*, this issue, 673–676.

Neville, M., Stutz, F., Lee, L., Davis, L.I., and Rosbash, M. (1997). The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr. Biol. *7*, 767–775.

Paxton, W., Connor, R.I., and Landau, N.R. (1993). Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. *67*, 7229–7237.

Piguet, V., Chen, Y.-L., Mangasarian, A., Foti, M., Carpenter, J.-L., and Trono, D. (1998). Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes. EMBO J. *17*, 2472–2481.

Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M.-A., Ratner, L., Lane, C.M., Moore, M.S., Blobel, G., and Bukrinsky, M. (1998). Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO. J. *17*, 909–917.

Re, F., Braaten, D., Franke, E.K., and Luban, J. (1995). Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G_2 by inhibiting the activation of $p34^{cdc2}$-Cyclin B. J. Virol. *69*, 6859–6864.

Simon, J.H.M., and Malim, M.H. (1996). The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. J. Virol. *70*, 5297–5305.

Simon, J.H.M., Miller, D.L., Fouchier, R.A.M., Soares, M.A., Peden, K.W.C., and Malim, M.H. (1998). The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission. EMBO J. *17*, 1259–1267.

Spina, C.A., Kwoh, T.J., Chowers, M.Y., Guatelli, J.C., and Richman, D.D. (1994). The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. *179*, 115–123.

Stade, K., Ford, C.S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041–1050.

Swingler, S., Gallay, P., Camaur, D., Song, J., Abo, A., and Trono, D. (1997). The Nef protein of human immunodeficiency virus type 1 enhances serine phosphorylation of the viral matrix. J. Virol. *71*, 4372–4377.

Vodicka, M.A., Koepp, D.M., Silver, P.A., and Emerman, M. (1998). HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. *12*, 175–185.

Wei, P., Garber, M.E., Fang, S.-M., Fischer, W.H., and Jones, K.A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell *92*, 451–462.

Willey, R.L., Maldarelli, F., Martin, M.A., and Strebel, K. (1992). Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. *66*, 7193–7200.

Yang, X., Herrmann, C.H., and Rice, A.P. (1996). The human immunodeficiency virus Tat proteins specifically associate with TAK in vivo and require the carboxyl-terminal domain of RNA polymerase II for function. J. Virol. *70*, 4576–4584.

Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M.B., and Price, D.H. (1997). Transcription elongation factor P-TEFb is required for HIV-1 Tat transactivation *in vitro*. Genes Dev. *11*, 2622–2632.