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ROLE OF VIRAL REGULATORY AND ACCESSORY PROTEINS IN HIV-1 REPLICATION

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1. ABSTRACT

Human immunodeficiency virus-1 (HIV-1) is the causative agent of acquired immune deficiency syndrome (AIDS), a disease characterized by $CD4^+$ T lymphocyte depletion. HIV-1 replicates actively in a variety of cells by encoding several regulatory (Tat and Rev) and accessory (Vpr, Vif, Vpu, and Nef) proteins. Accessory proteins, thought initially to be dispensable for infection, have now been shown to be important for efficient infection *in vivo*. Recent evidence suggests that certain viral proteins, like Vif, have evolved to overcome the antiviral mechanisms of the host, while proteins like Nef, which are markers for disease pathogenesis *in vivo*, help to increase pathogenesis by targeting bystander cells. Thus, these proteins control many aspects of the virus life cycle as well as host cell function, namely gene regulation and apoptosis. Understanding the mechanisms by which the virus is able to successfully replicate in host cells and subsequently cause gradual destruction of the immune system may yield new approaches for therapeutic strategies. In this review, we attempt to integrate information on the role of these regulatory and accessory proteins, emphasizing their interactions with other viral and cellular components, and the subsequent effect on viral replication.

2. INTRODUCTION

HIV-1 is a member of the lentivirus family of retroviruses and is the etiologic agent of AIDS. HIV-1 manipulates fundamental host cell processes in complex ways to achieve optimal replicative efficiency. The virus enters the cells via the envelope (Env) glycoprotein which binds the CD4 receptor along with the chemokine receptors CXCR4 or CCR5 (1-4). The viral RNA is then reverse transcribed into proviral DNA in the cytoplasm, enters the nucleus as a pre-integration complex, and is integrated into the cellular genome. HIV-1 replication is tightly regulated at the transcriptional level through the specific interaction of viral proteins, which bind to target sequences in the viral

RNA and profoundly affects viral expression. In addition to the structural proteins, Gag, Pol, and Env, the virus encodes two regulatory proteins - Tat (transcriptional transactivator) and Rev (regulator of virion gene expression). Tat is crucial for activated transcription from the HIV-1 long terminal repeat (LTR), while Rev modulates the transport of viral mRNAs from the nucleus to the cytoplasm (5-7). Besides these proteins, HIV-1 encodes four accessory proteins, Nef (negative effector), Vif (viral infectivity factor), Vpr (viral protein r), and Vpu (viral protein u). These are termed accessory or auxiliary proteins to reflect that they are dispensable for gene expression, even though they play an important role in pathogenesis and immune evasion (5, 7, 8). HIV-1 primarily targets and replicates effectively in $CD4^+$ T lymphocytes and to some extent in macrophages and dendritic cells. HIV-1 strains that preferentially use the CXCR4 co-receptor are termed T-tropic (X4), while those that use the CCR5 co-receptor are termed M-tropic (R5) strains. X4 strains can infect only T cells, R5 strains can infect macrophages, dendritic cells, and T cells, and X4R5 chimeric viruses can infect all cells susceptible to infection (9-11). The virus induces $CD4^+$ T cell depletion mainly by programmed cell death (apoptosis) (12). Tat, Nef, and Vpu proteins released from infected cells can induce apoptosis in uninfected bystander cells. Therefore, HIV-1 manipulates the host cell processes by a complex interplay of viral proteins at various stages of the viral life cycle and eventually causes apoptosis. For a more comprehensive review of other viral proteins, the reader is referred to the following references (5, 6). This review summarizes our current knowledge and understanding of the regulatory and accessory viral proteins and highlights their contribution in the regulation of HIV-1 replication.

3. TAT

HIV-1 encodes a potent transactivator, Tat, a 14 kD protein that is required for virus replication. In fact, Tat induces chromatin remodeling and recruits elongation-competent transcription complexes onto the HIV-1 LTR. Tat regulates viral gene expression by modulating the activity and association of cellular transcription factors with RNA polymerase II (RNAPII) at the 5' LTR (13-15). Hyperphosphorylated RNAPII is associated with the elongation complex, while hypophosphorylated RNAPII is recruited to promoters during formation of the pre-initiation complex (pre-IC). Tat associates with RNAPII complexes during early transcription initiation and elongation after promoter clearance and before synthesis of the full-length TAR RNA transcript (16). Tat regulates the expression of a number of cellular genes and plays a key role in AIDS progression and the development of AIDS-associated malignancies by interfering with cellular processes such as proliferation, differentiation, and apoptosis (17). In addition, Tat induces chromatin remodeling and recruits elongation-competent transcriptional complexes onto the HIV-1 LTR. Besides these functions, Tat is released from infected cells and interacts with different cell membrane-associated receptors such as the chemokine receptors (18, 19). Extracellular Tat is then internalized through active endocytosis (20, 21). The protein also has been shown to suppress reverse transcriptase (RT) activity at late stages of the viral life cycle, which restricts the premature reverse transcription of viral RNA in the cytoplasm and facilitates the packaging of an intact viral genome (22, 23). In the following sections the role of Tat in the modulation of gene expression will be discussed, as well as its extracellular effect on neighboring cells, and its role in apoptosis.

3.1. Transcriptional initiation

Tat transactivates HIV-1 by promoting transcriptional initiation and stabilizing elongation through its interaction with a number of viral, as well as cellular partners. HIV-1 LTRs contain a transactivation response element (TAR), a 59 residue RNA leader sequence located at the 5'-terminus. TAR extends downstream from the transcription initiation site and folds into a stable stem-loop RNA structure that is critical for Tat activation (14, 24, 25). Mutations introduced into the TAR structure lead to the accumulation of prematurely terminated viral transcripts, supporting the idea that an intact stem-loop in TAR is essential for trans-activation. Interaction of Tat with TAR leads to the enhancement of both transcription at the HIV-1 promoter and stabilization of the elongation complex (26-28). The transactivation role of Tat was shown to be enhanced by regulatory elements like AP1, Oct, Sp1, and NF-kappaB (29). Another study reported that alteration of the TATA motif also affected the inducibility of HIV transcription by Tat (30). Thus, these unique structural features, especially the NF-kappaB, SP1, and TATA elements present in the viral LTR, facilitate transcriptional activation by Tat.

An intact TATA element and functional TATA-binding protein (TBP) were found to be necessary for Tat association with the RNAPII holoenzyme (31, 32). Moreover, Tat regulates the specific binding of RNAPII to the TAR RNA (33). Thus, Tat binds to TAR and interacts with upstream regulatory elements to increase initiation complex formation on the HIV-1 promoter and stabilize complexes during elongation (figure 1). Some reports emphasize that Tat is recruited to the transcription complex through its association with TAR (14). However, increasing evidence highlights the involvement of Tat at the pre-initiation stage, prior to TAR binding by its association with components of the basal transcription machinery such as TFIIE, TFIIH, and TBP (31). In addition, Tat has been reported to interact with cellular transcription factors such as Sp1 (34, 35); TFIID (36); E2F-4, a member of the E2F family of transcription factors (17); Tat associated kinase (TAK) (37); and co-activators such as CBP/p300 (38) and P/CAF (39), further supporting its involvement at the pre-initiation stage and subsequently before the start of transcription elongation. In fact, protein-protein interaction of Tat with the cellular transcription factor Sp1 stimulates Tat transactivation (35, 40) and facilitates up-regulated expression from the HIV-1 LTR. Sp1-dependent Tat transactivation is also linked to cell cycle events where the LTR is activated by Tat in the G_1 phase in a TAR- and Sp1-dependent manner. However, in the G_2 phase, Tat is shown to activate the LTR in a TAR- and Sp1-independent manner. This latter mechanism is augmented by the accessory protein, Vpr, which arrests cells at the G_2/M

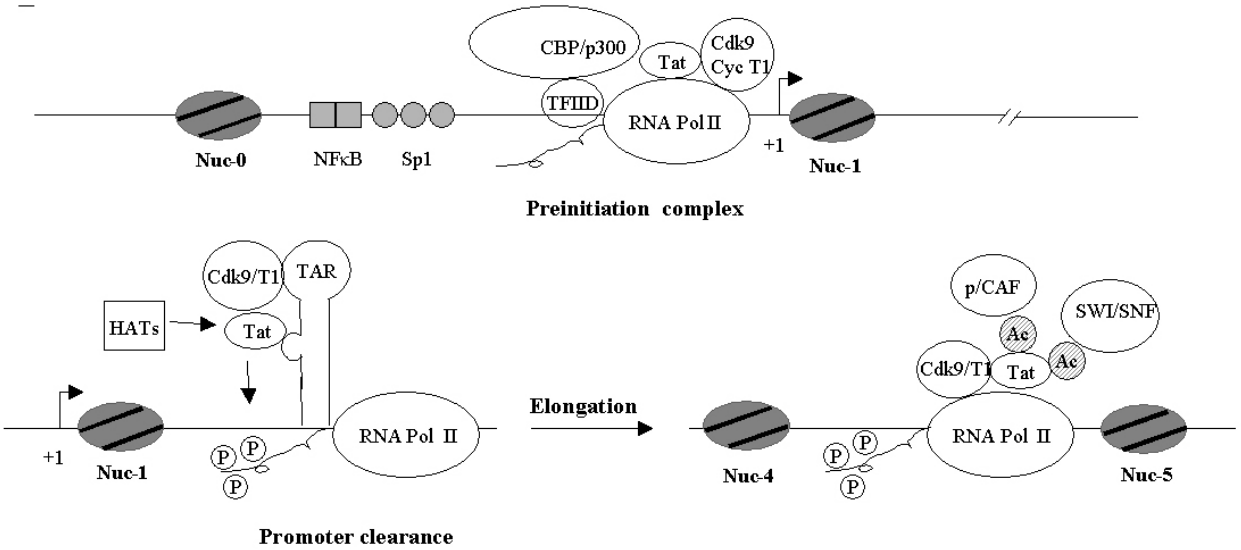

Figure 1. Transactivation by Tat. Transcription initiation begins with formation of the pre-initiation complex (pre-IC) on the HIV-1 LTR via Tat-mediated recruitment of various co-activator proteins, including CBP/p300 and P/CAF. Subsequently, Tat recruits CDK9 to the TAR structure, where CDK9 binds to the loop structure of TAR and phosphorylates the CTD of RNAPII. HATs then acetylate Tat, which promotes the dissociation of Tat from TAR, thereby stimulating elongation. Furthermore, acetylated Tat binds to the chromatin-remodeling complex SWI/SNF, which allows for the downstream regulation of nucleosome remodeling (80).

boundary (41). Interaction of Tat with the transcription factor NF-kappaB is also important for transcription from the viral promoter. Studies reveal that the RNA binding domain of Tat is dispensable for TAR-independent activation of the virus (42) and is mediated by the binding of NF-kappaB to the kappaB enhancer elements within the LTR (43, 44). Tat enhances the activation of NF-kappaB by stimulating degradation of the inhibitor, IkappaB-alpha (45) and increasing the capacity of NF-kappaB inducing kinase (NIK) to accelerate this degradation (46). NF-kappaB stimulates transcription initiation, though recently it has also been shown to stimulate transcription elongation from the HIV-1 LTR, and has the ability to disrupt chromatin structure through the recruitment of histone acetyltransferases (HATs) (47).

Although most of the research has focused on the ability of Tat to activate the HIV-1 LTR, increasing evidence indicate that Tat is also able to affect cellular gene expression. In a microarray experiment, de la Fuente *et al.* have observed that most of the cellular host genes in Tat expressing cells are down-regulated (48). This down-regulation was apparent in transcription of cellular receptors that have intrinsic receptor tyrosine kinase (RTK) activity, and signal transduction elements that mediate RTK function. For instance, Tat represses transcription of major histocompatibility class I (MHC-I) genes by interacting with the TAFII250 component of the general transcription factor, TFIID, and inhibiting its HAT activity. This might be one of the mechanisms by which the virus avoids immune surveillance (49). Tat has been shown to inhibit HAT, Tip60, and hinder the expression of cellular genes that normally interfere with viral replication (50). The interaction of Tat with HATs may also have consequences not only for transcription initiation, but also for elongation and chromatin remodeling processes.

3.2. Transcriptional elongation

In the absence of Tat, transcription from the HIV-1 LTR leads to the production of prematurely terminated viral transcripts due to the low processivity of RNAPII (14). Tat interacts with TAR, inducing modification of the chromatin structure at the HIV-1 LTR promoter, and stimulating the recruitment of elongation-competent RNAPII complexes (38). After binding to TAR RNA, Tat stimulates a specific protein kinase called Tat-activated kinase (TAK/pTEFb) (14, 51-53). It promotes elongation by recruiting C-terminal domain (CTD) kinases associated with the pre-IC, specifically TFIIH-associated cyclin-dependent kinase (CDK) 7 and pTEFb-associated CDK9, which hyperphosphorylate the CTD of RNAPII, thus increasing it's processivity (54). CDK9 is also the kinase in the TAK complex, which is analogous to a component of the pTEFb complex isolated from Drosophila (14). CDK9 can activate viral transcription when tethered to the heterologous Rev response element (RRE) RNA via Rev (55). Interaction of Tat with cyclin T1, the regulatory component of CDK9, results in the recruitment of the heterodimer cyclin T1/CDK9 to TAR, whereby it promotes elongation (figure 1). Formation of the ternary complex with cyclin T1 requires the functional TAR RNA loop sequence, which induces binding of pTEFb onto nascent HIV-1 TAR RNA (56-59). Thus, Tat-pTEFb complexes bind to TAR, where CDK9 phosphorylates RNAPII for efficient elongation of the viral genome. In addition, Tat inhibits a CTD phosphatase preventing the dephosphorylation of RNAPII CTD (15, 60). The stimulation of phosphorylation and inhibition of

dephosphorylation appears to be part of the transactivation function of Tat to promote transcriptional elongation.

Tat also associates with a T cell derived kinase (TTK), which contains CDK-activating kinase (CAK), a component of the transcription factor TFIIH (61). The TTK complex contains the CAK catalytic subunit, CDK7, as well as a CTD kinase that does not contain CDK7, cyclin H, and TFIIH. CAK activity is regulated either by phosphorylation of CDK7, or by the formation of a CDK7-cyclin H complex (62). The activation of CDK7 by Tat is considered to be one of the critical steps of Tat transactivation. TTK also contains another cellular kinase, CDK2, which is involved in Tat-mediated CTD phosphorylation, and is found associated with the RNAPII in the elongation complexes assembled on the HIV-1 LTR template. The dynamic association of Tat with cyclin E/CDK2 stimulates CTD phosphorylation by CDK2 (63). It is reported that cyclin E/CDK2 exhibits its maximum activity at the late G_1/S boundary. During apoptosis of infected cells, the loss of the G_1/S checkpoint is associated with loss of p21/Waf1 and increased activity of cyclin E/CDK2 (63). p21/Waf1 interacts with the CDK2/cyclin E complex and inhibits progression of cells into S phase. The loss of the G_1/S checkpoint in HIV-1-infected cells may partly be due to the ability of Tat to bind p53 (a known activator of the p21/Waf1 promoter) and to sequester its transactivation activity. This allows the host cells to enter the S phase and undergo subsequent processes such as RNA splicing, transport, translation, and packaging of viral DNA (64). Therefore, Tat induces a transition from abortive to productive transcription elongation and regulates cell cycle progression by interacting with cyclin/CDK complexes.

Furthermore, Tat stimulates co-transcriptional capping of HIV-1 mRNA shortly after transcription initiation by binding directly to the capping enzyme (65, 66). Tat transactivation and mRNA capping are correlated with RNAPII CTD phosphorylation, and the capping enzyme interacts specifically with the phosphorylated form of RNAPII CTD (67-70). In the absence of Tat, CDK9 phosphorylates serine 2 and CDK7 phosphorylates serine 5 of the RNAPII CTD, while CDK9 phosphorylates serine 5 in the presence of Tat (66, 71). Tat-mediated phosphorylation of serine 5 by CDK9 recruits the capping enzyme and stimulates its guanylyltransferase activity facilitating cap formation on the TAR stem loop RNA (65, 66). Fong *et al.* (72) demonstrated that TAT-SF1, which interacts with spliceosomal U small nuclear ribonucleoproteins (snRNPs), strongly stimulated polymerase elongation when directed to an intron-free HIV-1 template. TAT-SF1 was isolated as part of an elongation complex, linked to the C-terminus of Tat. Earlier studies showed that cellular proteins, such as Tat-SF1 and SPT5, were involved in Tat transactivation (73), and SPT5 interacted directly with the capping enzyme and enhanced mRNA capping (74). These results indicate that Tat-mediated phosphorylation of RNAPII CTD promotes transcriptional elongation and stimulates viral RNA capping.

In recent years, a number of studies have shown a promising set of inhibitors that can directly inhibit HIV-1 transcription. Pharmacological cyclin-dependent kinase inhibitors (PCIs), which bind to cyclin/CDK complexes and inhibit their activity reflect their promising role as antiviral agents in inhibiting HIV-1 (75, 76). Two PCIs, flavopiridol and roscovitine, have been found to be useful as antivirals (77). Flavopiridol can inhibit the cyclinT1/CDK9 complex (78) as well as CDK2 (79). Flavopiridol inhibits viral transcription via CDK9 and CDK2 inhibition and cell cycle progression via CDK2 suppression. Roscovitine is a purine analog effective against CDK2, -7 and -9, all of which have been described as Tat-interacting CDKs (75). Roscovitine selectively sensitizes both latent and activated infected cells to apoptose (75). These studies indicate that selective inhibition of CDKs by PCIs effectively disrupts Tat-dependent transactivation and thus viral replication.

3.3. Chromatin remodeling

The viral genome is packaged into higher order chromatin structure after it is integrated into the host genome (80). Tat can induce chromatin remodeling through the recruitment of chromatin modification and remodeling complexes onto the integrated viral LTR before the assembly of RNAPII complexes (21, 38). Hyperacetylation of core histones is correlated with transcription activation (81, 82), while hypoacetylation is correlated with transcription inhibition (83, 84), which results in the establishment of viral quiescence. After transcription activation by Tat, the integrated LTR becomes accessible to endonucleases and chromatin disruption occurs at the nucleosome (nuc-1) located at the transcription start site (85-87). The transcription factors YY1 and LSF recruit histone deacetylase 1 (HDAC1) to the HIV-1 LTR. It was shown that Tat is associated with histone acetylation at nuc-1 and down-modulation of HDAC1. Conversely, HDAC1 recruitment followed by hypoacetylation of nuc-1 may result in LTR repression (84). These studies suggest that nuc-1 plays a repressive role in HIV-1 transcription and histone acetylation may result in nuc-1 remodeling to overcome the inhibitory effect.

Acetylation of Tat establishes a novel protein-protein interaction domain on the surface of Tat that is necessary for the transcriptional activation of the HIV-1 promoter. Tat transactivation activity is dependent on lysine acetylation and its association with the nuclear HATs, p300/CBP (CREB binding protein), p300/CBP-associated factor (P/CAF), and GCN5 (88-90), which are capable of regulating the interaction of nucleosomes and chromatin remodeling complexes (38). The major targets of acetylation by p300 are lysine residues (Lys50 and Lys51) in the arginine-rich motif used by Tat to bind RNA and for nuclear import (91). P/CAF acetylates Tat on Lys28, which enhances the interaction of Tat with pTEFb (92). Tat also influences acetylation of NF-kappaB (p50 subunit) mediated by CBP/p300, which aids CBP/p300 to acquire new partners and enhance the DNA binding properties of these factors on the LTR, thus increasing the rate of transcription (93). Tat has also been shown to form a ternary complex with P/CAF and p300 and increase affinity for the CDK9/pTEFb CTD kinase complex (94). The bromodomain of P/CAF binds specifically to Tat acetylated at Lys50 and this interaction leads to dissociation of Tat

from TAR and serves to transfer Tat onto the elongating RNAPII complex (95-98).

However, Tat acetylated at Lys28 does not bind to the bromodomain of P/CAF, but instead binds to pTEFb, resulting in increased phosphorylation of the CTD (99). To identify cellular proteins that differentially interact with unmodified or acetylated Tat and characterize their functional significance during Tat transactivation, affinity purification and mass spectrometry were performed on pull-down protein complexes from human T cell extracts with immobilized acetylated or unacetylated Tat peptides (80). The proteins identified include cyclin T1, P/CAF, and TRIM family proteins, which were previously shown to interact with Tat. The other proteins include transcription-associated proteins (IKKE, TIF-1a, DDX3, SCL, zing finger protein 140, and ITF-2), signal transduction proteins (mitogen-activated protein kinase (MAPK) 4 and protein tyrosine phosphatase), cell cycle-associated proteins (CDC7-like and PITSLRE), histone/chromatin-associated proteins (P/CAF, RCC1, P/CAF-associated factor 400, and CHD2), and proteins with an unknown function. Acetylated Tat was shown to bind more to P/CAF, SWI/SNF, and IKKalpha as compared to unmodified Tat (80). These observations suggest that the unmodified Tat is involved in transcription initiation, and acetylated Tat dissociates from TAR and recruits bromodomain-containing protein complexes such as SWI/SNF, P/CAF, and the histone kinase IKKalpha to facilitate elongation from the HIV-1 chromatin template. Interestingly, it has been shown that Tat can autoacetylate at lysine residues 41 and 71 and peptides lacking these two lysines cannot enhance the HAT activity of p300 (94). Collectively, these findings indicate that transcriptional activation occurs through regulation of chromatin conformation and that Tat largely utilizes cellular acetylation and deacetylation signaling systems to enhance viral gene expression and modulate the expression of cellular genes. The modulation of cellular genes allows Tat to influence many cellular pathways, including apoptosis.

3.4. Role of Tat in apoptosis

Tat plays a critical role in the depletion of $CD4^+$ T cells and peripheral blood mononuclear cells (PBMCs) by stimulating a cascade of events resulting in the up-regulation of the potent immunosuppressive cytokine, transforming growth factor-beta (TGF-beta) (100). Tat promotes apoptosis by inducing tumor necrosis factor-related apoptosis-induced ligand (TRAIL) in PBMCs, which can destroy uninfected bystander cells (101, 102), and by targeting the microtubule network (103). Tat stimulates a mitochondria-dependent apoptotic pathway by binding to tubulin/microtubules through a four amino-acid subdomain of its conserved core region. In addition, Tat can cause the activation of Bim, a pro-apoptotic Bcl-2 factor and a transducer of death signals (103). It was also shown that Tat-transfected cells express increased amounts of Bax, a Bcl-2 family protein known to induce apoptosis (104). Moreover, Tat interacts with the Egr family of transcription factors, Egr-2 and –3, which are major participants in the up-regulation of the Fas ligand (FasL) promoter. Increased expression of FasL may contribute to the apoptosis of T cells (105).

Tat has also been shown to be involved in HIV-1-associated dementia (HAD) by causing apoptosis of neurons. Tat disrupts neuronal calcium homeostasis by perturbing calcium-regulating systems (106) and increases oxidative stress in a mechanism involving the combined effects of tumor necrosis factor (TNF)-alpha and Tat (107). Recently, it was also found that an isoform of growth factor receptor bound protein (Grb2), Grb3-3, a signaling molecule associated with the MAPK pathway, is involved in apoptosis. Tat and Nef can independently up-regulate Grb3-3 expression, which is followed by cell death (108). The up-regulation of FLICE/caspase-8 by Tat may also contribute to increased apoptosis (109). Furthermore, in PBMCs, Tat induces IL-10, which has immunosuppressive activity and is implicated in viral propagation, via the protein kinase C pathway (110). Tat can therefore induce apoptosis by transcriptionally activating several apoptotic proteins, significantly contributing to the immunopathogenesis of AIDS. As previously discussed, Tat can modulate viral and cellular gene expression in infected cells and ultimately induces apoptosis by disrupting several mechanisms in immune cells as well as neurons. Moreover, Tat is also secreted by HIV-1 infected cells and affects the neighboring uninfected environment.

3.5. Extracellular Tat

Tat is released from infected cells and taken up by uninfected cells (111, 112). Extracellular Tat was shown to enter cells mainly due to the presence of a cluster of basic amino acids, stimulating the transcriptional activity of the HIV-1 LTR and cellular genes (113, 114). This internalization property of Tat can be used for cellular delivery of proteins fused or cross-linked to Tat into different cell types, thus acting as a means for transcellular protein transduction. In fact, Tat was shown to increase the delivery of plasmid DNA or other large biomolecules to the nuclei of cells by transduction (115, 116). Tat acts as a pleiotropic molecule inducing biological effects on target cells by adsorptive endocytosis and by interacting with cell surface receptors (21, 114, 117). Extracellular Tat has an effect on gene expression, growth, and induction of angiogenic activity in endothelial cells by interacting with the vascular endothelial growth factor (VEGF) receptor-2 (Flk-1/KDR), and stimulation of monocyte migration (118, 119). Internalization of Tat requires cell surface heparan sulfate proteoglycans that act as receptors for extracellular Tat (120, 121). Tat exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. Tat can also enhance the chemotactic and invasive behaviors of monocytes by recruiting monocytes into extravascular tissues, a process that may contribute to the destruction of tissues and cellular processes (122, 123). Finally, Tat promotes chemotaxis by inducing the chemokine receptors CCR5 and CXCR4 and promoting infection with both M-tropic and T-tropic HIV-1 strains in leukocytes (19, 124, 125). Collectively, these data suggest that Tat up-regulates monocyte adhesion to the endothelium and contributes to destruction of tissues.

4. REV

Rev is an 18 kD phosphoprotein involved in the nucleocytoplasmic transport of viral RNAs. It forms

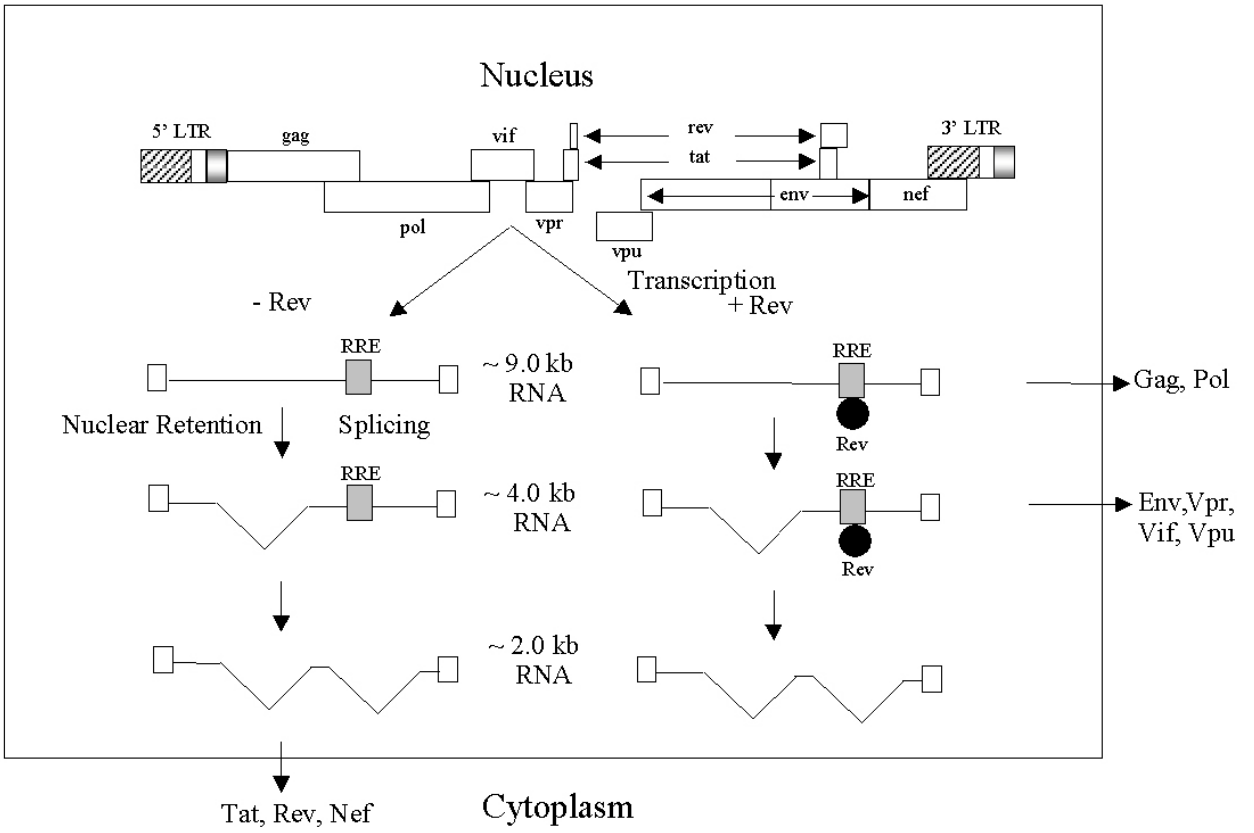

Figure 2. Rev mediated mRNA export from nucleus to cytoplasm. The ~9.0 kb primary transcript undergoes splicing to generate different singly and multiply spliced mRNAs. The ~2.0 kb viral RNAs are fully spliced and encode the Tat, Rev, and Nef proteins. The singly spliced ~4.0 kb RNA encodes Env, Vif, Vpr, and Vpu, and the unspliced ~9.0 kb transcript encodes Gag and Pol, and is also packaged into progeny virions. Rev binds to the RRE present in the env open reading frame (ORF) and facilitates nuclear transport. During the early phase of infection, in the absence of Rev, only the ~2.0 kb transcripts can enter the cytoplasm, while the unspliced transcripts are retained in the nucleus. During the late phase of infection, Rev promotes nuclear export and enables the cytoplasmic expression of singly spliced and unspliced transcripts.

multimers, binds to the Rev response element (RRE), and most importantly, promotes the nuclear export of unspliced viral mRNAs, particularly the intron containing ~9 kb and ~4 kb mRNAs. Rev contains an arginine-rich sequence in its N-terminal domain that serves as its nuclear localization signal (NLS) (126) and RNA-binding domain (RBD) (127). The NLS is critical for Rev function, since Rev is expressed in the cytoplasm and must enter the nucleus for its mRNA export function. The RRE is a 351 nucleotide RNA structure that is present within the env intron in all unspliced and singly spliced viral mRNAs and bound by Rev's RBD (128). The sequences on either side of this domain confer its multimerization properties, yielding the possibility of eight or more Revs binding to a single RRE (129, 130). Casein kinase II has been shown to phosphorylate Rev at two residues, Ser5 and Ser8, thereby enhancing Rev-RRE binding (131, 132). On the other hand, the leucine-rich C-terminal domain contains a nuclear export signal (NES) (133), and mutational analysis of this region demonstrated that the C-terminal domain confers the transactivator function of Rev (134). Furthermore, the nuclear diffusion inhibitory domain (NIS) has been shown to play an important role in maintaining an increased number of Rev proteins in the nucleus, while conferring intracellular stability (135). Interestingly, Rev has been shown to accumulate in the cytoplasm upon transcriptional termination via inhibition of RNAPI and -II, possibly due to changes in Rev phosphorylation (136).

HIV-1 transcription yields ~2 kb, ~4 kb, and ~9 kb RNAs. The ~2 kb transcript is fully spliced and encodes Tat, Nef, and Rev transcripts, while the partially spliced ~4 kb RNA encodes Env, Vif, Vpr, and Vpu transcripts. The ~9 kb transcript is the full-length unspliced transcript, and, in addition to other viral proteins, contains Gag and Pol. Therefore, during the early stages of infection, which correspond to no or low levels of Rev, only the ~2 kb transcripts are able to pass through the nuclear membrane and enter the cytoplasm, while the unspliced transcripts remain in the nucleus. Upon maturation in the cytoplasm, Rev is able to enter the nucleus via interaction with importin-beta. During the late phase of infection, corresponding to higher levels of Rev, Rev is able to direct the nuclear export of larger transcripts and ensure proper splicing (figure 2). In this way, Rev is essential for

supplying the necessary viral factors for viral replication, maturation, and pathogenesis.

Rev has been shown to bind to a number of cellular proteins including the eukaryotic initiation factor 5A (eIF-5A) (137); importin-beta (138); nucleoporin-like proteins Rip/Rab (139, 140) and NLP-1 (141); the nucleolar protein B23 (142); the splicing factor ASF/SF2-associated protein p32 (143); the Src-associated protein in mitosis (Sam68) (144); a nuclear kinesin-like protein, REBP (145); Nup98 (146); and CRM/exportin 1 (147). All known Rev-interactors contribute to the nuclear export of viral mRNA species. There have also been other cellular proteins, including the nucleoporins that comprise the nuclear pore complex (NPC) and Sam-like proteins, that have been elucidated as Rev co-factors. It was recently reported that Rev binds to a purine loop in the SL1 region of the HIV-1 leader RNA, which has a nearly identical structure to the RRE (148).

While the interaction between importin-beta and the Rev NLS is necessary for the nuclear import of Rev, the interaction between the CRM/exportin 1 transport receptor and the Rev NES is required for the Rev-mediated export of viral mRNAs. eIF-5A is essential for the efficient interaction of Rev and CRM (149), which interacts with the nucleoporins CAN/nup214, nup153, nup98, nup62, and nuclear actin. Rev displacement from importin-beta, mediated by the RanGTPase cycle, frees the NLS/RBD to allow for RRE binding (150). Following Rev-RRE-CRM association, this complex migrates to the NPC, where the various nucleoporins bind Rev and allow translocation into the cytoplasm.

Interestingly, an early study showed that the binding of a Rev monomer to the RRE is unable to export a small ribonucleoprotein (RNP) complex (150). This finding implies the importance of Rev multimerization in mRNA export. Likewise, it is also possible that multiple CRMs may be needed for efficient export of viral mRNAs. A recent study illustrates that, through the direct tethering of CRM to viral mRNAs via a heterologous RBD, Rev function can be bypassed (151). Another report showed that the over-expression of Sam68 functionally substitutes for Rev function and can also synergize with Rev (152). On the presumption that alternative splicing and/or nuclear retention of mRNA species may contribute to viral latency, Pongoski et al. (153) elucidated various SR (serine-arginine) proteins that affected Rev function. They found that both SF2/ASF and SC35 proteins inhibited Rev function, while hTra2alpha, an SR-related protein, promoted Rev function. Importantly, these functions were also found to be dose-dependent and cell line-dependent. Deletion of the exon splicing enhancer (ESE) found in tat exon 3 and the exon splicing silencer (ESS) were found to enhance and inhibit the Rev-mediated nuclear export of viral RNAs, respectively. These findings, coupled with the biphasic nature of Rev regulation on viral RNA export, implicate Rev in viral latency. Conversely, the next discussed viral protein, Nef, plays an active role in promoting viral replication and infectivity.

5. NEF

The Nef gene encodes a 205 amino acid, myristoylated phosphoprotein, which plays a critical role in viral replication and pathogenesis. Nef is considered a critical marker for disease pathogenesis *in vivo*, since loss of Nef function results in patients exhibiting low viral loads and delayed or absent progression to AIDS (154-156). Initially, it was suggested that Nef represses viral replication by inhibiting transcription from the HIV-1 LTR, hence its description as a 'negative effector' (157, 158). Later, it was reported to be crucial for high-titer viral replication. Nef mediates its effects after viral entry and before the completion of reverse transcription indicating that Nef likely interacts with an internal component of the HIV-1 core, facilitating an early step in infection following the dissociation of the viral capsid in the target cell (159). Nef is incorporated into HIV-1 particles and is cleaved by the viral protease; however, cleavage of Nef is not required for the enhancement of infectivity (160, 161). Studies have shown that both the myristoylation signal and a N-terminal cluster of basic amino acids are required for virion incorporation and for plasma membrane targeting of Nef (162). Nef influences the activation state of the host cell enhancing the host cell ability to support viral replication by functioning as an adaptor bringing together different host cells proteins, protein kinases, and components of the endocytic machinery. The major functions of Nef include down-regulation of cell surface CD4 and MHC-I molecules, and the ability to augment virus infectivity and modulate cellular signaling pathways in lymphocytes and macrophages (163-166).

5.1. Down-regulation of CD4

Nef down-regulates the cell surface expression of CD4 receptors early in HIV-1 infection through rapid endocytosis and decreases transport of CD4 from the Golgi complex to the plasma membrane, influencing virion infectivity and the severity of T cell depletion (164, 166-168). Nef renders these effects by disrupting the CD4-p56(lck) complex on the cell surface to allow CD4 internalization and diversion of the internalized CD4 to a lysosomal pathway for its degradation, likely through a phosphatidylinositol-3-kinase (PI3K) pathway (169). Furthermore, Nef interacts with several downstream cellular partners including the adaptor complex of clathrin-coated pits and the beta subunit of COP-I coatomer (170) to down-regulate CD4.

Nef interacts with a di-leucine motif in the cytoplasmic tail of CD4 and enhances the endocytosis of CD4 through clathrin-coated pits (168, 171, 172). Nef bridges CD4 with the medium (mu) chains of the adaptor protein complex (AP) and this interaction is important for CD4 down-modulation. Adaptor complexes are structures that recruit clathrin to the cytoplasmic tail of receptors containing internalization signals (173). Nef also interacts with Nef binding protein 1 (NBP1), which is similar to V1H, the regulatory subunit of the vacuolar membrane ATPase (174). This interaction links Nef to the endocytic machinery and facilitates internalization. V1H binds to the C-terminal flexible loop in Nef and to the medium chain of AP-2, functioning as an adaptor for interactions between Nef and AP-2 (175).

Another downstream partner of Nef is the beta subunit of COP I coatomers. Nef targets internalized CD4

molecules for degradation by acting as a connector between CD4 and the beta-COP I coatomers in endosomes. An acidic dipeptide in Nef is responsible for beta-COP I recruitment and routing CD4 to lysosomes (176). These findings demonstrate that Nef acts as a bridge between CD4 and intracellular trafficking pathways, targeting the receptor for degradation in the lysosome. The manipulation of intracellular trafficking proteins may be a common mechanism by which Nef aids the virus, as further illustrated below by the down-regulation of MHC-I molecules. Furthermore, decreased expression of surface CD4 would prevent super-infection as well as suppress the T cell response in infected individuals.

5.2. Down-regulation of MHC-I

Another important function of Nef is to reduce cell surface levels of MHC-I molecules to protect infected cells from cytotoxic T lymphocyte recognition and to enable immune evasion by HIV-1. Nef induces the internalization of MHC-I molecules facilitating their accumulation in endosomal vesicles and subsequent degradation. In the presence of Nef, MHC-I molecules are re-localized from the cell surface to the trans-Golgi network (TGN), which requires the binding of Nef to PACS-1, a molecule that controls TGN localization of the cellular protein furin (177). Nef and PACS-1 seize the ARF6 endocytic pathway by a PI3K-dependent process to down-regulate cell surface MHC-I to the TGN (178). The motifs in Nef, which are essential for this mechanism include the acidic cluster, a SH3 domain binding site, and the N-terminal alpha helix (179).

Nef down-regulates a subset of MHC-I (HLA-A and HLA-B) molecules and enables these proteins to accumulate in the Golgi and co-localize with clathrin-coated vesicles. It was shown that Nef forms complexes with MHC-I by binding directly to the cytoplasmic tail of these proteins via a tyrosine-based sorting signal. Nef interacts with the medium subunit of the AP complex involved in the recognition of the tyrosine-based sorting signal, facilitating the connection between MHC-I and clathrin-dependent endocytosis (180). The remaining HLA-C and HLA-E molecules prevent recognition by natural killer cells that attack cells expressing small amounts of MHC-1 (181). Nef, therefore, functions as an adaptor molecule in linking MHC-I to cellular trafficking proteins. The down-regulation and degradation of HLA-A and HLA-B, while maintaining the expression of HLA-C and HLA-E, would allow infected cells to evade the immune response.

5.3. Signal transduction interference

Nef plays a critical role in the modulation and control of signal transduction elements and cascades. It can perturb signaling pathways by interacting with several signaling molecules including a serine/threonine kinase called the Nef-associated kinase (NAK), a member of the p21-activated kinase (PAK) family (164, 182), and also with members of the Src-family of tyrosine kinases (165, 183), such as Lck (184), Hck (164, 185), Fyn (186), MAPK (187), and protein kinase C (188). Nef binds to a subset of the Src family of kinases via its polyproline type II helix (Pxx) 4 motifs and binds to the SH3 domain of Hck, stimulating its tyrosine kinase activity (189). Nef also interacts with both the SH2 and SH3 domains of the T lymphocyte Lck protein, leading to impaired cell signaling (185). Studies have shown that Nef also forms a complex with Fyn and is involved in T cell perturbation by altering T cell receptor signaling (186). Nef induces both the DNA binding and transcriptional activities of the activator protein, AP-1, which is likely to be mediated via the MAPK (ERK1 and 2) signaling pathway and requires the proline-rich motif of Nef (190). These results indicate that Nef binds to several protein kinases and may perturb T cell function.

Nef has also been shown to activate NAK (PAK2) via the guanine nucleotide exchange factor Vav and the small GTPases Rac1 and Cdc42 (191). Nef mediated activation of PAK involves PI3K, which acts upstream of PAK and this Nef-associated PI3-PAK complex phosphorylates the pro-apoptotic Bad protein blocking apoptosis. These anti-apoptotic effects of Nef may be one of the likely mechanisms in promoting efficient viral replication and pathogenesis (192). Nef alters T cell activation and signaling by up-regulating the expression of both Fas (CD95) and FasL on the surface of infected cells (193), which leads to the killing of bystander cells. Nef is thought to induce FasL by interacting specifically with the zeta chain of the TCR complex (194). Nef binds to and inhibits apoptosis signal-regulating kinase 1 (ASK1), a serine/threonine kinase, which is a key signaling intermediate in the Fas and TNF-alpha death-signaling pathways. The interaction with ASK1 inhibits both Fas-mediated and TNF-alpha-mediated apoptosis, and the activation of the downstream c-Jun amino-terminal kinase. It has been suggested that Nef strategically promotes bystander cell killing through the induction of FasL, while simultaneously protecting the infected host cells from the same pro-apoptotic signals through its interference with ASK1 function (195).

It has been demonstrated that Nef also interacts with the inositol trisphosphate receptor (IP_3R) residing in the endoplasmic reticulum (ER) to activate calcium signaling and promote T cell activation by inducing IL-2 expression and subsequent autocrine stimulation mediated by the transcription factor nuclear factor of activated T cells (NFAT) (196). This event involves activation of calcineurin by changes in calcium metabolism induced by Nef (197), which results in increased IL-2 production from T cells stimulated by CD3 or CD28 and requires the myristoylation signal and SH3-binding proline-based motif of Nef. The Nef-mediated induction of IL-2 involves the activation of both NFAT and NF-kappaB (198). Nef also disrupts T cell receptor (TCR) machinery by altering the signaling of CD3-TCR. It down-regulates CD28, a co-stimulatory receptor that mediates T cell activation, by accelerating CD28 endocytosis. Studies show that CD28 co-localizes with a clathrin adaptor and hence it appears that Nef down-modulates CD28 via the AP-2 pathway, which involves a complex of Nef, AP-2 and CD28. This event is likely to disrupt antigen-specific signaling in infected T cells (199).

Nef, along with several proteins involved in T cell signaling, associate with membrane microdomains known as lipid rafts to activate T cells (198). Nef may interfere with TCR-mediated activation by regulating intracellular trafficking and clustering of GM1-enriched microdomains at the cell surface. A functional link among GM1-enriched lipid microdomains, Vav, an essential regulator of actin cytoskeletal rearrangement, and Nef has been described. Nef can decrease the level of plasma membrane GM1 in unstimulated T cells affecting the behavior of infected cells towards antigen recognition, and Vav towards counteracting such an effect. This down-regulation is associated with the inhibition of NFAT activation (200). In addition, Nef interferes with signal transducer and activator of transcription 1 (STAT1)-mediated intracellular signaling in monocytes/macrophages. It activates the alpha and beta isoforms of STAT1, followed by increased expression of STAT1 and interferon regulatory factor-1 (IRF-1), a transcription factor regulated by STAT1 activation (201). Nef also activates STAT3, which targets Nef to the late endosomes/lysosomes. This mechanism is mediated by the release of soluble factors, including MIP-1alpha, suggesting that the intervention of Nef in intracellular signaling may generate a secondary wave of activation that could be significant in viral pathogenesis (202). These findings indicate that Nef interferes with intracellular signal transduction through its interaction with various critical cellular proteins, resulting in the perturbation of immune cell function. This perturbation may be one cause of the dysfunction of the immune response observed in HIV infected patients.

5.4. Role of Nef in enhancing viral infectivity

Nef plays a crucial role in the early phase of the virus replication cycle, from virion adsorption to integration. Nef stimulates virion infectivity in the producer cell, although its effect is manifested in the subsequent target cell (203). Virions produced in the absence of Nef have been found to be inefficient in the completion of viral DNA synthesis and exhibit a severe defect in viral entry into cells. Nef acts through modulation of viral particles to enhance virus infectivity in a cell-dependent manner (204). It allows the induction of components required for completing steps that follow entry, leading to efficient reverse transcription of the viral genome, highlighting the Nef-mediated stimulation of proviral DNA synthesis, and its role in boosting viral infectivity (205). Nef is required for efficient viral entry, incorporation of Env into virions, and viral infectivity (206). HIV buds from lipid rafts and requires cholesterol for its egress from and entry into cells. Nef increases the biosynthesis of lipid rafts and enriches viral particles with newly synthesized cholesterol. It binds cholesterol and transports it to the site of viral budding, providing essential building blocks for the formation of viruses that replicate optimally in the host (207), thus promoting viral morphogenesis.

Nef also physically interacts with Tat, which results not only in the enhancement of Tat-induced HIV-1 LTR-mediated gene expression but also in virus production and progression of disease (208). Nef, like Tat, is also shown to interact with p53 via its N-terminus and inhibit p53 DNA binding activity and transactivation, thereby protecting cells against p53-mediated apoptosis. Inhibition of p53 activity may increase HIV replication by preventing p53-mediated apoptosis and extending the life of infected cells (209). In addition, Nef inhibits the endocytosis of DC-SIGN [dendritic cell (DC)-specific lectin], resulting in increased surface expression of DC-SIGN and clustering of DCs with T cells. DC-SIGN mediates clustering of DCs with T lymphocytes, which is crucial to the initiation of an immune response. DC-SIGN also binds HIV Env glycoproteins to allow capture of HIV virions by DCs. The capture of HIV virions and T cell clustering by DCs would also increase HIV transmission (210). The actions of Nef which boost virion infectivity include the transportation of cholesterol to sites of viral budding and the increased surface expression of DC-SIGN.

6. VPR

Vpr is a 15 kD regulatory protein packaged in the HIV-1 virion and is assumed to be responsible for viral replication and pathogenesis in the early stages of infection. After a retrovirus enters the cell, the viral RNA genome is reverse transcribed in the cytoplasm. For integration and subsequent replication and propagation, the viral DNA and associated viral proteins need to enter the nucleus. The two main functions of Vpr are transport of the pre-integration complex (PIC) and, later in infection, induction of G_2 arrest. Additionally, Vpr is involved in nuclear localization, apoptosis, and transactivation of the HIV-1 LTR and other cellular promoters (211-215). Vpr incorporation into the virion is mediated by the CTD of the Pr55 (Gag) polyprotein precursor, which contains the NCp7 protein responsible for genomic RNA encapsidation, and the p6 protein needed to induce Vpr encapsidation in HIV-1 (216). Vpr is also a nucleic acid binding protein (217) and Vpr-DNA complexes enter the cells through endocytosis. The nucleic acid binding activity of the protein is mediated by amino acids in the C-terminal region, and provides a means for understanding the molecular interactions between the virus and host cells (218). In addition, Vpr inhibits IL-12 production by enhancing glucocorticoid activity, thereby suppressing innate and cellular immunities of infected individuals (219). The functions of Vpr will be described in more detail below.

6.1. Nuclear transport of the pre-integration complex

Vpr shows a predominant role in long-term disease manifestation by inducing viral infection in non-dividing cells such as monocytes and macrophages (220, 221). HIV-1 replication in non-dividing cells depends on the import of the viral PIC into the cell nucleus. The PIC contains three nucleophilic proteins, matrix, integrase, and Vpr, all of which are involved in nuclear targeting. The nucleocytoplasmic shuttling of Vpr not only contributes to nuclear import of the PIC but also enables Vpr to be present in the cytoplasm for incorporation into virions, leading to the spread of the virus to non-dividing cells (222).

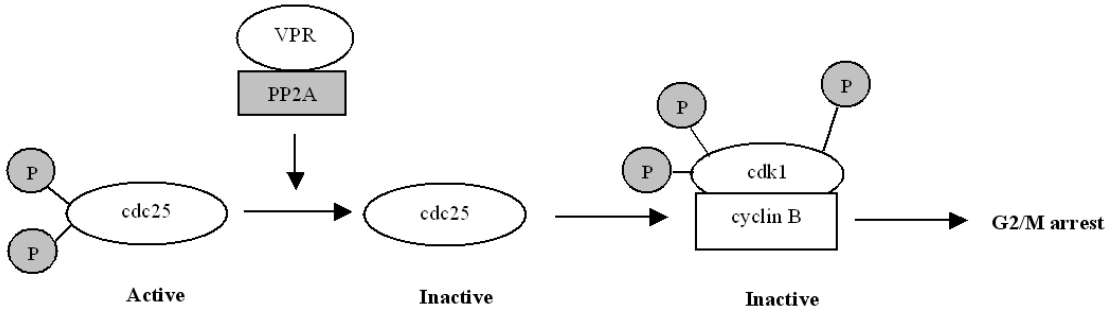

Figure 3. Cell cycle G_2/M arrest mediated by Vpr. In infected cells, Vpr mediates G_2 arrest by enhancing the nuclear import of PP2A and regulating its enzymatic activity towards active phosphorylated nuclear cdc25. The C-terminus of Vpr associates with the B55-alpha regulatory subunit of PP2A, an upstream regulator of cdc25. This interaction increases the catalytic activity of PP2A, targets the complex to the nucleus where active cdc25 is located, and results in the dephosphorylation of cdc25. The inactive cdc25 keeps cdk1/cyclin B in its hyperphosphorylated form, thereby causing G_2 arrest (231).

Early in infection, the nuclear localization property of Vpr helps to target the proviral DNA to the host cell nucleus (223). Vpr functions in conjunction with the Gag matrix protein to facilitate the import of the PIC (224). Nuclear import of the virus is mediated by an interaction between NLSs present on the matrix and integrase proteins, and the cellular NLS receptor, karyopherin-alpha. Vpr increases the affinity of this interaction, thus enhancing translocation of the PIC (225). The protein shuttles between the nucleus and the cytoplasm, but a significant fraction is concentrated in the nuclear envelope so that Vpr can interact with components of the nuclear pore complex. Interaction between HIV-1 Vpr and human nucleoporin CG1 (hCG1) has been observed in the yeast two-hybrid system. hCG1 plays a role in the docking of Vpr at the nuclear envelope and this association may contribute to the disruption of the nuclear envelope leading to the nuclear import of the viral DNA (226, 227). Furthermore, hVIP/mov34 (human Vpr interacting protein) is a Vpr ligand and a member of the nucleocytoplasmic shuttling family of proteins. While Vpr does not enter the nucleus with the PIC, it does utilize the hVIP/mov34 protein for transport into the nucleus (228). These studies indicate that Vpr plays a major role in nuclear import by interacting with karyopherin-alpha and in modulating the nuclear pore complex.

6.2. G_2 cell cycle arrest

Vpr induces G_2 cell cycle arrest in proliferating cells and prolonged G_2 arrest leads to apoptosis. Cell cycle arrest is mediated by the C-terminal basic domain of Vpr (229, 230). Vpr suppresses activation of mitotic CDK and thereby prevents infected cells from undergoing mitosis and proliferation. The ability of Vpr to arrest cells at the G_2 phase of the cell cycle is due to inactivation of the cdk1/cyclin B complex and its upstream regulator cdc25, resulting in a decrease in the phosphorylation of substrates involved in cell cycle progression from G_2 to mitosis. Vpr also forms a complex with protein phosphatase 2A (PP2A), an upstream regulator of cdc25, through a specific interaction with the B55 regulatory subunit of PP2A, thereby enhancing the recruitment of PP2A to the nucleus and dephosphorylation of the cdc25 substrate. The inactivation of cdc25 leads to the inactivation of cdk1/cyclin B and ultimately to G_2 arrest (figure 3). This phenomenon suggests that Vpr induces G_2 arrest by enhancing nuclear import of PP2A and regulating its activity towards phosphorylated nuclear cdc25 (231).

Finally, Vpr also activates the expression of the CDK inhibitor p21/Waf1/Cip1. This activation is observed in p53 positive cells but not in p53 null cells. Furthermore, Vpr and p53 have an additive effect on p21 gene transcription, resulting in the arrest of cells at the G_2/M phase of the cell cycle (232). G_2 arrest induced by Vpr provides additional time needed for protein synthesis and packaging of the virion in order to produce a competent virus. Vpr is also taken up from the extracellular medium and is efficiently imported into the nucleus of transduced cells. It can effectively transduce through intact cytoplasmic membranes, leading to G_2 cell cycle arrest and apoptosis in bystander cells (211), thereby enhancing the pathological effects of the virus.

6.3. Transactivation

Vpr-mediated enhancement of viral replication is associated with transactivation of the HIV-1 LTR. The transactivating properties of Vpr are conserved throughout evolution in primate lentiviruses (215). Vpr transactivates sequences present within nucleotides -278 to -176 of the LTR (233) and enhances transcriptional activity of the HIV-1 LTR by interacting with Tat and cyclin T1/CDK9 (234) and with the cellular proteins Sp1 and p53, all of which control LTR activation at early stages of infection (235). Other studies also show that Vpr increases IL-8 expression in primary T cells and macrophages by activating transcription factors NF-kappaB and NF-IL-6 (236). Vpr cooperates with p300/CBP, co-activators which regulate NF-kappaB dependent transcription and are critical and necessary for HIV-1 LTR activation (237). Furthermore, Vpr directly binds to the C-terminal domain of p300/CBP to enhance Tat-induced HIV-1 LTR promoter activity (238). Vpr thereby promotes transcription by acting as an adapter linking transcription components and co-activators.

Vpr was also shown to activate virus production by inducing TNF, a proinflammatory cytokine, which is known to activate HIV-1 expression and replication (239). Vpr stimulates transcription of genes lacking a common

DNA target sequence by interacting with and changing the conformation of the basal transcription factor, TFIIB (240). Vpr has also been shown to interact with a cytoplasmic protein, Vpr-binding protein (VprBP), which retains Vpr exclusively in the cytoplasm, possible bringing about changes in the host cell cytoplasm that affect viral replication (213). In addition, Vpr also causes up-regulation of cyclic AMP response element (CRE) directed transcription and increases the phosphorylation of CREB (241). Vpr can also regulate 3' end mRNA processing. The Poly(A) polymerase enzyme responsible for poly(A) addition to primary transcripts contains multiple phosphorylation sites for cdk1/cyclin B kinase which regulate its catalytic activity (242). As Vpr inhibits cdk1/cyclin B, it appears that Vpr is involved in transcription by mediating processes such as polyadenylation of mRNA transcripts. Collectively, these results provide evidence for cell cycle-independent regulation of gene expression by Vpr.

6.4. Role of Vpr in Apoptosis

Vpr has been shown to promote and inhibit apoptosis. In the absence of T cell receptor mediated activation, Vpr induces apoptosis, whereas following T cell receptor activation, Vpr inhibits apoptosis. Vpr regulates apoptosis by suppressing NF-kappaB activity via the induction of its inhibitor, IkappaB and suppresses the expression of IL-2, IL-4, IL-10, IL-12, and TNF-alpha, which are NF-kappaB-dependent (243, 244). In addition, Vpr promotes the up-regulation of Bcl-2 and other apoptotic genes in the mitochondrial apoptotic pathway resulting in neuronal apoptosis, which may be a mechanism for HIV-1 induced encephalopathy (245). Prolonged G_2 arrest induced by Vpr results in the depletion of Wee1, a cell cycle regulatory kinase, indicating that Wee1 may be a key regulator of Vpr-mediated apoptosis (246). Vpr also forms cation-selective ion channels in planar lipid bilayers and is able to depolarize neurons causing an inward sodium current that may result in cell death (247).

Most importantly, Vpr induces mitochondrial membrane permeabilization by interacting with a transition pore complex, which is comprised of the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane (248). Vpr-ANT interaction is important in inducing apoptosis (248-250). Vpr enters the intermembrane space through VDAC and binds to the intermembrane face of ANT causing inner mitochondrial membrane swelling and rupture of the outer membrane (251). Bax, a pro-apoptotic factor of the Bcl-2 family, enhances the channel activity of Vpr-ANT and localizes to the outer mitochondrial membrane causing Vpr and Bax to induce outer mitochondrial membrane permeabilization (252). The ability of Vpr to depolarize mitochondria membrane potential results in the release of apoptotic proteins such as cytochrome C, which in turn leads to the activation of caspase 9 culminating in the activation of the caspase 3 pathway and downstream events leading to apoptosis (253).

Low Vpr concentration at the onset of infection inhibits the host apoptotic response and helps to prolong viral replication (254, 255). The anti-apoptotic effects of Vpr are exerted in bystander cells and may lead to the preservation of a pool of infected cells facilitating persistence and subsequent spread of the virus (251). Moreover, the constitutive expression of Vpr causes up-regulation of the bcl-2 oncogene, which also has anti-apoptotic activities (256). These studies demonstrate that Vpr exhibits a dual role in regulating apoptosis in infected $CD4^+$ T cells.

7. VIF

The use of a *vif* mutant HIV-1 virion has proven extremely valuable for understanding Vif's contribution to viral infectivity (257-260). The major cell types for infection, namely $CD4^+$ T cells (primary and several T cell lines) and macrophages, are restrictive (non-permissive) for HIV-1 replication and require Vif for maintaining viral infectivity (261-263). Initial studies suggested that Vif was important for early events after virus entry but preceding or during the early stages of viral DNA synthesis, since *vif* mutants exhibited reduced RT activity *in vitro* and synthesis of early and late DNA products (257, 264). Vif was observed to bind to the viral genomic RNA (264, 265); however the mechanism in which Vif contributed to reverse transcription has only begun to be clarified.

Vif is a 23 kD basic protein that is expressed late during infection in a Rev-dependent manner (266) and is largely localized within the cytoplasm (267, 268). Vif exists in a soluble cytosolic form that co-localizes with the intermediate filament vimentin (268) and can be found associated with intracellular membranes via its C-terminus, which is also essential for its function and multimerization (267, 269). Early reports found that Ser144, Thr155, and Thr188 are all phosphorylated *in vivo* by an as yet unidentified cellular kinase (270, 271). A later study observed that Thr96 and Ser165 are also phosphorylated by the p44/42 mitogen-activated protein kinase (also known as ERK1 and ERK2) (271). The importance of phosphorylation on Vif activity is exemplified when mutation of the phosphorylation site at Ser144 to an alanine resulted in a loss of activity and an almost complete inhibition of viral replication (270). By means of extensive deletion and substitution analysis of Vif, Fujita *et al.* (272) discovered that residues 88 and 89 within the hydrophilic region are critical for HIV-1 replication in nonpermissive cells due to the enhancement of the steady-state expression of Vif. Interestingly, a recent report by Mehle *et al.* (273) observed that Vif was modified by ubiquitination suggesting another possible route that may influence the function of Vif.

Low levels of Vif (1 to 40 molecules/virion) have been found to be packaged into newly synthesized progeny (268, 274, 275); however the significance of this event is not entirely clear. *vif* mutant HIV-1 virions exhibit structural abnormalities within the cone-shaped core. Electron-dense material was observed to be compacted at the broad end of the cone, while the narrow end appeared transparent (276). A recent report indicates that these *vif*-defective cores were more susceptible to disruption by

detergents, high salt, pH, and RNase than wildtype virion cores (277). Vif increases the stability of virion cores, which may prevent premature degradation upon viral entry (277). Conversely, the level of Vif incorporation does not appear to modulate its function or relate to virion infectivity, though it did correlate with productive and not chronic infection (278). The effect of Vif on nonpermissive cells, where it is critical for proviral DNA synthesis (258), is highly dependent on cellular factors with which Vif associates.

Vif has been shown to bind to a number of cellular proteins, including Hck tyrosine kinase (Hck) (279); a component of the HIV-1 PIC, Ku70 (280); Sp140 nuclear protein (280); HP68 when bound to Gag (281); spermine/spermidine N1-acetyl-transferase (SSAT); vimentin; Triad 3; and a novel Vif-binding protein (NVBP) (282). It has also been shown that Vif binds to HIV-1 genomic RNA in the cytoplasm and forms a 40S mRNP complex that most likely mediates viral RNA interaction with HIV-1 Gag precursors (265). On a similar note, the HP68 protein is essential for the post-translational events in immature capsid assembly and may therefore be another route for Vif incorporation into virions (281). Conversely, Hck is a tyrosine kinase that has been shown to inhibit HIV-1 production and consequent infectivity in vif-defective virus, while having no effect on the wild-type virus (279). Therefore, Hck has cellular antiviral properties in nonpermissive cells, which are subverted in the presence of Vif. The Ku70 protein, which is a component of the PIC, may allow for the recruitment of Vif to initiate proviral DNA synthesis. Since SSAT is involved in polyamine metabolism and polyamines are important in the reverse transcription process, the Vif-SSAT interaction most likely increases the efficiency of reverse transcription (282). Sp140 is an IFN-gamma-inducible protein that is both lymphocyte- and macrophage-specific and almost exclusively localized to nuclear bodies (283). Interestingly, it has been shown that Sp140 is found in all non-permissive cells and that Vif is able to partially localize Sp140 to the cytoplasm from the nucleus (280). Though the data is not conclusive, Sp140 may confer nonpermissive cells with an ability to inhibit viral replication in the absence of Vif relative to permissive cells.

Shortly after the identification of Sp140, another group elucidated a cellular protein termed CEM15, which was later identified as the apolipoprotein B mRNA editing enzyme [APOBEC-3G (284)]. CEM15 was shown to be a cytidine deaminase capable of inducing a guanine to adenine hypermutation in the plus-strand of newly synthesized viral DNA (285, 286). This finding was extremely significant with respect to HIV-1 replication because of the high frequency of mutations during reverse transcription, which has been accepted as one of the main driving forces in viral escape from immune cells, as well as the generation of drug-resistant strains. Since the main cell types infected by HIV-1 are nonpermissive cells, the presence of Vif most likely prevents the hypermutation activities of CEM15 and allows for the minimal introduction of mutations during reverse transcription. On the other hand, it is probable that in the presence of attenuated Vif and/or low levels of Vif, this endogenous inhibitor of HIV-1 may also serve to increase the variability of viral proteins and strains present during infection. Though there have been conflicting reports on the level and importance of Vif incorporation into virions, a recent report demonstrates that Vif forms a complex with human CEM15 and not mouse CEM15 and prevents its encapsidation into newly synthesized virions (287). This may be another plausible explanation for the restriction of HIV-1 to humans and not mouse cells. Furthermore, Stopak *et al.* (288) have recently shown that Vif prevents CEM15 encapsidation by impairing its translation, as well as hastening its post-translational degradation through ubiquitination and targeting to the 26S proteasome via its SLQ(Y/F)LA motif (289, 290). The antagonistic interaction of Vif with APOBEC-3G appears to be a promising avenue for rational drug design (291-293). While Vif is important in the later stages of the viral life cycle, Vpu is also important at late stages aiding in the release of infectious virions.

8. VPU

Vpu is a 17 kD viral protein containing a hydrophobic membrane anchor and a phosphorylated cytoplasmic tail (294). It is an integral membrane protein that forms ion channels in planar lipid bilayers and enhances the permeability of the plasma membrane to several molecules (295). Vpu performs two major functions in the viral life cycle; it induces the degradation of CD4 receptors and enhances the release of virions from infected cells (296, 297). Along with Tat and Vpr, Vpu has also been shown to contribute to apoptosis. The reader is referred to several excellent reviews specifically dealing with the structure/function relationship of Vpu in HIV infection (294, 298, 299).

8.1. Degradation of CD4

Vpu is involved in the down-modulation of the CD4 receptor during viral infection, which is a multi-step process dependent on the phosphorylation of the cytoplasmic tail of Vpu and subsequent binding to CD4-gp160 complexes (300-302). Casein kinase 2 phosphorylates Vpu at Ser52 and Ser56 in the acidic dodecapeptide region of the cytoplasmic domain (301, 303). CD4 has been shown to bind to Env (gp160) leading to sequestration of this complex within the ER (304). In the absence of Vpu, CD4 receptors were found to accumulate at the cell surface and be efficiently recruited into virions (305). Vpu binds to and reduces the formation of CD4-gp160 complexes, causing an increase in gp160 maturation, trafficking, and incorporation into nascent viral particles (306). Vpu also interacts with a F box protein, betaTrCP, a component of the E3 ubiquitin ligase complex and allows for polyubiquitination of CD4 molecules (307). This finding suggests that Vpu induces degradation of CD4 via the ubiquitin-proteasome pathway (308-312). Therefore, Vpu-mediated degradation of CD4 facilitates the release of Env glycoproteins from the ER and prevents surface expression of CD4, which would interfere with both virus release (313) and the infectivity of the particles produced (314). Further studies have shown that Vpu

down-regulates MHC-I molecules on the surface of the infected host cells (308, 315) by retaining nascent MHC-I chains within the ER (316). In this way, Vpu, like Nef, helps infected cells evade detection from the immune system. Interestingly, betaTrCP has been shown to promote the degradation of various cellular substrates including IkappaB-alpha, an inhibitor of NF-kappaB, resulting in decreased NF-kappaB activity. This may provide a unique mechanism in which Vpu affects NF-kappaB mediated gene expression (317).

8.2. Release of viral particles

Since Vpu diminishes CD4 cell surface expression and packaging into the virion, this will allow the release of viral particles containing functional Env glycoprotein capable of binding CD4 molecules at the surface of target cells (305). Thus promotion of CD4 proteolysis by Vpu helps to release fully infectious virions. Ubp, the Vpu-binding protein, interacts with both Vpu and Gag and plays a key role in Vpu-mediated enhancement of viral particle release (318, 319). Studies show that the effect of Vpu on efficient particle release depends on the rate of cell proliferation. Cells arrested by contact inhibition or cell cycle arresting agents have shown dependence on Vpu for efficient particle release. In contrast, actively proliferating cells do not exhibit enhanced particle release with Vpu expression, demonstrating that efficient viral release in quiescent cells requires the presence of Vpu (320). Furthermore, Vpu can form ion channels by oligomerization of Vpu via it's transmembrane domain (294, 321). However, how this may lead to increased viral particle formation has not been explored.

8.3. Role of Vpu in apoptosis

Vpu directly contributes to the induction of apoptosis in HIV-1 infected $CD4^+$ T cells, and Vpu-mediated apoptosis has been shown to be independent of other viral proteins (322). As described above for CD4 degradation, Vpu interacts with betaTrCP, a protein involved in the degradation of cellular proteins including IkappaB, and can stimulate the induction of apoptosis in infected T cells. Vpu down-modulates the NF-kappaB-dependent anti-apoptotic factors Bcl-xL, A1/Bfl-1, and the TNF receptor-associated factor 1 (TRAF1), and concurrently increases the levels of active caspase-3. Since NF-kappaB regulates chemokines, Vpu can have a severe impact on disease progression by inhibiting chemokine production (322). Vpu is also reported to increase the sensitivity of infected cells to Fas induced death (323); however, the underlying mechanism is unclear. These results suggest that Vpu promotes apoptosis through activation of the caspase pathway and inhibition of NF-kappaB-dependent anti-apoptotic genes.

9. SUMMARY AND PERSPECTIVES

HIV-1 is a complex retrovirus encoding several regulatory and accessory proteins that function at various stages of the viral life cycle. The requirement of accessory proteins for the development of AIDS and persistence of these viral sequences in various isolates reflects their importance to HIV-1 infection *in vivo*. While most experimental systems to date have focused on these proteins individually there is evidence to suggest that these proteins work in concert to increase HIV infectivity and pathogenesis.

After entry into the host cell, the newly reverse transcribed provirus integrates into the genome and is organized into a repressive chromatin structure. However, before these later steps occur, virion-associated Vpr, Nef, and Vif help increase viral infectivity. The importance of Vif in the life cycle of HIV-1 is best demonstrated by the phenotype of *vif*-defective virions within non-permissive cells, i.e. reduced levels of viral DNA synthesis and production of highly unstable replication intermediates. This phenotype may be explained in part by the presence of APOBEC-3G/CEM15, a cytidine deaminase that hypermutates viral DNA rendering it inactive in non-permissive cells. Vif inactivates APOBEC-3G by targeting it for ubiquitin/proteasomal degradation. This targeted degradation would help to decrease APOBEC-3G within newly infected cells and increase viral infectivity. Whether Vif targets other proteins for degradation is unknown at this time, yet these observations suggest that Vif evolved as a mechanism against antiviral defenses. Nef, like Vif, is incorporated into the virion, suggesting that some of its functions occur soon after entry. Nef was found to down-regulate surface CD4 and MHC I receptors soon after infection by two distinct mechanisms. First, CD4 associated with AP-2 via Nef, thus targeting these molecules to endosomes for subsequent lysosomal degradation. Second, Nef also links MHC I (HLA-A and HLA-B) to the endocytic pathway redirecting these proteins to the TGN. Down-regulation of these molecules is thought to prevent superinfection, which would lead to premature death of infected cells, in the case of CD4, and evasion from immune surveillance, in the case of MHC I. Thus, Nef and Vif, acting as adaptor molecules, could be the first means by which HIV-1 deregulates existing antiviral mechanisms.

Conversely, Vpr acts at several levels to ensure that the proviral genome integrates into the cellular genome. While Vpr is associated with the pre-integration complex in the cytoplasm, Vpr is not translocated into the nucleus with the complex. Instead, Vpr first facilitates the binding of karyopherin alpha to the Matrix (MA) NLS. Secondly, Vpr stabilizes the association between nucleoporins and the import complex at the nuclear envelope (NE) by preventing disassociation of the karyopherin alpha and PIC complex upon nucleoporin binding. Recent reports have shown that Vpr also interacts with nucleoporins; in particular, Vpr was shown to bind to hCG1. However, this interaction between Vpr and nucleoporins might be transient and take place as Vpr, either alone or in the context of PICs, is translocated or acts at the NPC. Further studies are needed to resolve this issue. Interestingly, Vpr binding at the NPC causes herniations and transient ruptures of the NE. Thus, the association of Vpr with hCG1 may cause alterations of the NE architecture that aid in translocation of PICs into the nucleus. A novel mode in which Vpr may promote integration of the proviral genome is by inducing a

transient G_2/M arrest. Certain non-homologous end-joining (NHEJ) enzymes have been shown to be more active at S/G_2 phase (324). Although initially thought to be important at later steps of viral replication, blockage of cells at G_2 could allow for NHEJ repair of double strand breaks that occur as a result of proviral integration.

Before activation of the virus, Nef, Vpu, and Tat act to disrupt cellular signaling pathways. First, Vpu targets CD4 and MHC I molecules for proteasomal degradation and retention within the ER, respectively. Virion-associated Nef acts initially by removing these molecules from the membrane, while newly synthesized Vpu operates in the ER to maintain their absence from the cell surface. The additional advantages of decreasing CD4 levels are maturation of Env/gp160 molecules, increasing infectivity of the nascent virion, and augmenting Nef's ability to control signal transduction pathways, as described earlier. Tat, while important for viral transcription, also alters cellular transcription by decreasing expression of serine/threonine receptor tyrosine kinases and components of the MAPK cascade, which control proliferative and/or differentiation signals. By limiting amounts of certain key factors, Tat permits Nef to have a greater ability to manipulate these signaling cascades by competing out interactions between host factors. Collectively, these viral factors help to isolate infected cells from extracellular signaling that may compromise the viral life cycle.

Upon activation, high titer viral replication is dependent on the viral protein, Tat and Tat-associated complexes. Early during initiation, Tat recruits HATs to the 5' LTR promoter to destabilize the repressive nucleosomal structure. Tat-mediated histone acetylation is required for the further recruitment of SWI/SNF chromatin remodeling complexes and transcription factors. Interestingly, targeted acetylation of other factors, including NF-kappaB and Tat itself, has been shown to increase DNA and histone binding, respectively. Evidence from our own group has suggested that acetylation of Tat results in a change of partners, including acquisition of the SWI/SNF complex. Furthermore, ubiquitination of Tat has also been demonstrated both *in vitro* and *in vivo*, which increased Tat transactivation. Consequently, Tat activity may be regulated post-translationally permitting for the transition between initiation to elongation. Vpr and Nef also have been demonstrated to synergistically increase Tat-dependent transcription. Vpr increases interaction of Tat with HAT complexes and 3' mRNA processing via disruption of the cyclin B/cdk1-mediated inhibition of polyadenylation. Although not fully understood, Nef has been demonstrated to increase LTR activation *in vivo*. Nef can bind to Tat directly; however, the exact contribution of this interaction to Tat transactivation is not known at this time. Nef was observed to inhibit p53 DNA binding and transactivation. Since p53 negatively regulates the HIV-1 promoter, Nef is thus able to help Tat block p53 inhibition.

Recently, it has become more apparent that transcription and RNA processing are coupled events. In addition to enhancing the recruitment of chromatin remodeling factors and transcription factors, Tat increases co-transcriptional capping through cyclin T1/cdk9 phosphorylation of the RNAPII CTD. This phosphorylation leads to loss of SPT5 binding to the CTD, which results in two potential outcomes. Free SPT5, would be able to bind to the capping enzyme and stimulate RNA guanylyltransferase (RGT) activity and increase Tat activation of the HIV-1 LTR through promoter clearance. Whether other accessory proteins, such as Vpr and Nef, are directly or indirectly involved in mRNA processing, such as splicing or capping, is unknown at this time. Conversely, Rev was thought to inhibit or repress splicing. However, this may be a consequence of Rev efficiently mediating the nuclear export of unspliced and singly spliced viral mRNAs from the nucleus to the cytoplasm, decreasing the pool of mRNAs available for splicing. The nuclear export of viral mRNAs is dependent on the binding of Rev to the RRE and nuclear export/import shuttle proteins, such as Crm1. Recently, Sam68 has been shown to augment and substitute for Rev function. Sam68 associates with Rev and the RRE and is thought to contribute to docking of the Rev/RRE/CRM1/RanGTP complex to the nuclear pore for translocation. While its role in Rev-mediated transport is not entirely understood, the importance of Sam68 has been shown by siRNA experiments, where targeted degradation of Sam68 resulted in inhibition of HIV-1 production. Therefore, these regulatory and accessory proteins synergistically activate transcription and RNA processing.

During the last stages of replication, Vif, Nef, and Vpu contribute to virion release and infectivity of subsequent target cells. Nef has been shown to target neighboring dendritic cells by inhibiting endocytosis of DC-SIGN. This increases both the clustering of infected T cells with dendritic cells and transmission of the virus. In this way, Nef triggers early events of infection in target cells. Likewise, increased cholesterol content within the membrane of progeny virion by Nef is thought to facilitate the formation of the complex between gp120, CD4, and CXCR4. In addition to decreasing CD4 expression, Vpu (acting as an ion channel) could enhance virus particle formation via alteration of membrane potential. However, further studies are needed to resolve this issue. Vif is thought to increase virion core stability since vif-defective virions were observed to contain abnormal core structures. Vif is a substrate for the viral protease within virions, which could increase the ability of Vif to facilitate virion stability/maturation.

Lastly, Tat, Vpr, and Vpu have been found to contribute to apoptosis either through altered expression of pro- and anti-apoptotic proteins or through inducing mitochondrial membrane permeabilization. Limited levels of these viral proteins early in infection and during latency might help to reduce the potential of latently infected cells apoptosing without high titer virus production. However, upon activation, the levels of these proteins would increase to levels sufficient to initiate apoptosis. It may be argued that the morphological features of apoptosis decrease the potential of virion budding since it has been shown that within apoptosing cells, the cytoskeletal network (e.g. actin and myosin) reorganizes into a peripheral ring to prepare

for blebbing (325). However, G_2/M blockage by Vpr and/or disruption of GTPase signaling pathways by Nef may help to delay commitment to apoptosis or extend certain phases of apoptosis, respectively, to allow sufficient time for viral replication. This is exemplified by the fact that isolated HIV-1 particles were demonstrated to contain cytoskeletal architecture proteins such as actin, cofilin, and moesin (326).

In conclusion, while in many cases the molecular function of these viral protein are not fully understood, there is an agreement that these proteins act as multifunctional adapters, subverting cellular processes to increase the efficiency of viral replication. Focusing on how these viral regulatory and accessory proteins regulate cellular factors and are themselves regulated, with respect to post-translational modifications and cellular binding partners, promises to provide unique targets for future antiretroviral therapies.

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