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MINIREVIEW

Molecular Biology of the Human Immunodeficiency Virus Accessory Proteins

RAMU A. SUBBRAMANIAN AND ERIC A. COHEN*

Laboratory of Human Retrovirology, Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada H3C 3J7

The human immunodeficiency virus (HIV) is the causative agent of AIDS. HIV belongs to a unique virus family, the *Retroviridae*, a group of small, enveloped, positive-strand RNA viruses. These viruses code for the enzyme reverse transcriptase, which enables them to replicate their RNA genome through a DNA intermediate. Simple retroviruses contain three long, contiguous open reading frames (ORFs) coding for the *gag*, *pol*, and *env* proteins, which constitute their structural and enzymatic repertoire, all packaged in the progeny virion. However, HIV belongs to the lentivirus subfamily, members of which are characterized by several additional ORFs not found in simple retroviruses. These ORFs code for viral proteins usually not packaged in the virion but readily detectable in the cells. Much evidence indicating that these gene products, collectively referred to as auxiliary proteins, are capable of regulating viral replication and infectivity has accumulated in the past decade.

HIV type 1 (HIV-1) possesses at least six such auxiliary proteins, namely, Vif, Vpr, Tat, Rev, Vpu, and Nef. The Tat, Rev, and Nef proteins are synthesized early from Rev-independent multiply spliced mRNAs (≈2 kb), while Vif, Vpr, and Vpu are expressed late from Rev-dependent singly spliced mRNAs (≈4 kb). The closely related HIV-2 does not code for Vpu, though it codes for another late protein, Vpx, not found in HIV-1 (Fig. 1). Mutations affecting either Tat or Rev severely impair viral replication, indicating that these two auxiliary proteins are essential for viral replication (reviewed in reference 9). In contrast, mutations affecting other auxiliary proteins do not greatly perturb the viral replication kinetics, at least in vitro. Hence, these proteins have been dubbed dispensable or nonessential for in vitro replication and are usually referred to as accessory gene products.

Though accessory proteins are not required for viral replication, they are nonetheless capable of modulating replication events, even in vitro, and accordingly, phenotypes associated with their expression have been recognized. Importantly, accumulating evidence suggests that these proteins may modulate viral pathogenesis in vivo, thus affecting disease progression and outcome. The focus of this review is on the dispensable auxiliary protein subset, consisting of Vif, Vpr, Vpx, Vpu, and Nef, whose function, at least in vitro, appears to be accessory and not essential.

* Corresponding author. Mailing address: Laboratory of Human Retrovirology, Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, C.P. 6128, Station Centre-Ville, Montreal, Quebec, Canada H3C 3J7.

Vif

The phenotype associated with the 23-kDa accessory protein Vif is currently well established. "Vif" stands for viral infectivity factor and stems from early functional studies that demonstrated the virions generated in the absence of this protein to be as much as a 1,000 times less efficient in establishing an infection (12, 54). In comparison, cell-to-cell infection was slightly impaired but was not as dramatically affected, indicating that the defect lies primarily in the progeny virions generated in the absence of Vif. This phenotype is particularly interesting, as Vif does not appear to be incorporated in the virion (12, 53, 61). A varying requirement for Vif in transformed cells has been documented, ranging from severe restriction to slight impairment of infectivity, depending on the cell type used (11, 14, 48, 61). However, a pronounced replicative defect is the norm in primary cells and is probably more representative of the Vif-negative phenotype that exists in vivo (11, 14). The varying requirement for the protein in certain cell lines may reflect the presence or absence of cellular substitutes which presumably are not available in primary cells.

To explain how Vif, a protein not readily detectable in the virions, affects infectivity, it has been suggested that the protein may affect the processivity or incorporation of other virion-associated proteins such as the envelope gene products (19, 48). However, subsequent studies have failed to document a notable difference in either the quantity, processivity, or the apparent molecular weight of envelope and other viral proteins from virions generated in the absence of Vif and, hence, do not support the notion of Vif affecting viral entry (14, 39, 61). Rather, it appears that the proviral synthesis following entry was inefficient in these viruses, though the reverse transcriptase from these virions was capable of efficiently initiating DNA synthesis (53, 61).

From a mechanistic point of view, it is conceivable that Vif acts late during virion assembly and the effects are manifested only later in the viral life cycle. A recent study indicates that Vif may be necessary for proper packing of the viral nucleoprotein core (27). Though the mechanism behind this effect is currently not known, a model based on such a function can accommodate many confirmed observations regarding Vif phenotype. For example, it has been demonstrated that, in cell types in which Vif is required for the production of infectious virus, the protein is required at the time of virus production, suggesting that it is likely to play a role during virus assembly or maturation (14). Also, Vif has been demonstrated to be a cytoplasmic protein capable of tight membrane association, where, presumably, it can function during the final stages of viral assembly and morphogenesis (17, 41). Impairment of proviral synthesis documented in some studies may result from the improper nucleoprotein core maturation, which may affect

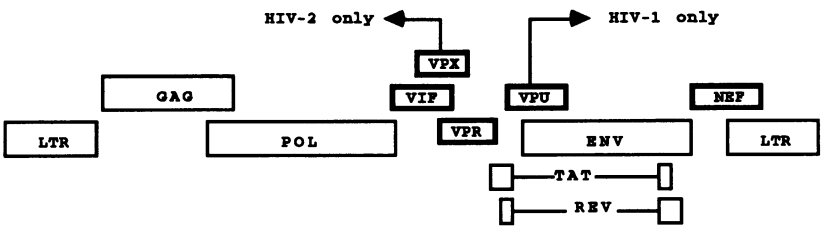

FIG. 1. Genomic organization of HIV. The approximate genomic positions of the accessory proteins discussed in this review are indicated. Vpu is unique to HIV-1, and the Vpx ORF is present only in HIV-2. Some degree of overlap in the reading frames is a regular feature, especially in the 3′ and 5′ ends of the ORFs. Note that Vpu overlaps Env and that Nef extends well into the 3′ long terminal repeat (LTR).

efficient uncoating and/or reverse transcription. Further advancement in our understanding of Vif phenotype will require more biochemical studies geared to identify the viral and/or cellular factors which are targeted by Vif during virion assembly.

Vpr

The accessory protein Vpr (viral protein R) is a 14-kDa gene product that confers rapid growth advantage to viruses expressing the protein (7, 45). Vpr-positive strains grow faster and produce moderately higher levels of virus than their Vpr-negative counterparts. This augmentation of virion production is more pronounced in primary macrophages in both HIV-1 and HIV-2 systems, suggesting that Vpr function may be important in specific target cells (24, 62). Interestingly, the protein does not appear to confer notable growth advantage in primary T cells (4). Vpr is unique among the accessory proteins in that it is assembled in the virion most likely through interaction with the Gag polyprotein precursor p55 (6, 34). Absence or truncation of p6 (a protein, like Vpr, unique to lentiviruses, located at the distal end of the Gag precursor) prevents Vpr incorporation into the virion (46). The presence or absence of envelope and genomic RNA does not appear to affect Vpr incorporation (34, 46). Recently, Lu et al. demonstrated that at the cellular level HIV-1 Vpr is localized predominantly in the nucleus (38).

The virion association of Vpr is highly indicative of its participation in early events during viral replication. Virion-associated nonstructural proteins in many viral systems play pivotal enzymatic functions in early replication steps, because cellular homologs either are unavailable or are sequestered, for example, in the nucleus. It is possible that Vpr is one such protein capable of augmenting early virus-specific functions such as reverse transcription, stabilization of RNA-DNA or DNA-DNA structures, migration of the proviral DNA complex to the nucleus, or integration (6). A role for Vpr at the preintegration level is supported by a recent report that identifies Vpr as one of the redundant viral nucleophilic determinants (the other being the matrix protein p17) that ensures efficient nuclear import of the preintegration complexes in nondividing cells such as macrophages (25).

In addition, experimental evidence also suggests that Vpr may function at the level of gene expression early in the infection. Vpr is localized primarily in the nucleus and demonstrates moderate transactivating ability from both HIV long terminal repeat and heterologous promoters in in vitro assays. However, the exact mechanism by which Vpr augments protein synthesis is not known (7). Vpr may alter cellular gene expression to foster a milieu that can both initiate and sustain efficient viral replication such as regulation of cellular activation and differentiation. It is interesting in this regard that Vpr induces differentiation of a rhabdomyosarcoma cell line (36). Such transcriptional activity, most likely mediated through cellular factors may promote basal long terminal repeat activity immediately following integration, a period during which the Tat protein is unavailable. Also, one cannot rule out the possibility that such transcriptional activity promotes viral replication following the early events. Though our understanding of the functional and biochemical mechanisms of this protein is at its infancy, some evidence indicates an important role for Vpr in vivo: rhesus monkeys infected with Vpr mutants of simian immunodeficiency virus (SIV) reverted to functional ORFs in vivo. Also, this study found high virus loads in the monkeys which reverted to functional Vpr (33).

Vpx

The 12- to 16-kDa Vpx gene is unique to HIV-2 and SIVs and is not present in HIV-1 (Fig. 1) (26). However, it shares a strong homology with HIV-1 Vpr and is similarly conserved in HIV-2 and most SIV groups (59). In addition to their unique Vpx, HIV-2 and most SIVs also possess the actual Vpr ORF, overlapping Vpx sequences (Fig. 1). It has been suggested that Vpx could have originated from the duplication of Vpr in these viruses (59). Like Vpr, Vpx is also packaged into the mature virion and has been shown to confer a rapid-growth advantage to viruses expressing the protein (29, 67).

In terms of a functional mechanism, on the basis of Vpr-Vpx sequence homology, virion incorporation, and apparently similar phenotypes (specifically, the ability to confer the rapid-growth advantage), it is tempting to speculate that Vpx and Vpr affect coordinately and synergistically the early events. This proposition is supported by some experimental evidence, as Vpx appears to augment early proviral synthesis in infected peripheral blood mononuclear cells while current evidence suggests a role for Vpr in the events immediately following reverse transcription (7, 25, 29). A role for Vpx at the level of reverse transcription is supported by an earlier study suggesting that the protein interacts with single-stranded RNA (26). Virion localization of Vpx suggests that it is closely associated with the exterior p17 matrix and not with the core structure (67). In this regard, it is interesting that the matrix protein and Vpr are both involved in nuclear import of the proviral DNA complex, events that immediately follow reverse transcription. However, how exactly Vpx affects reverse transcription is currently unclear.

Vpu

Vpu (viral protein U) is an accessory protein unique to HIV-1, and no analogous proteins exist in HIV-2 or most of the SIVs. The primary phenotype demonstrated by the 16-kDa Vpu is one of efficient release or export of virions from cells expressing the protein (8, 55–57). Also, absence of Vpu

resulted in virions containing multiple cores and in viral budding into vacuolar compartments as opposed to the plasma membrane (32, 65). Hence, expression of Vpu is associated with the proper maturation and targeting of the virions and with their efficient release. Another well-documented effect of Vpu expression is the reduction of syncytium-mediated cytopathicity, presumably due to the efficient release of virions and the lack of accumulation of viral proteins at the cell surface (57, 65). However, some evidence suggests that the reduction of cytopathic effect by syncytium formation may at least in part stem from a separate effect, as some mutants of Vpu, in spite of efficient viral release, showed no reduction of syncytial killing (13).

More recently, Vpu has been demonstrated to degrade CD4 in the endoplasmic reticulum (ER). This effect is functionally relevant to the virus, as CD4 traps viral envelope precursors in the ER, thus affecting their transport to the cell surface (63, 64). The degradative effect of Vpu was CD4 specific, as Vpu did not mediate degradation of CD8 similarly retained in the ER (35). Vpu-specific response sequences appear to be present in the cytoplasmic domain of CD4, and the degradative effect is dependent on Vpu being associated with the same membrane compartment as CD4 (5, 35, 40, 60). The facilitation of virion release and the effect on CD4 are not related events, as virion release was not dependent on the envelope or CD4 expression (66). While Vpu-mediated degradation of CD4 occurs in the ER, Vpu-mediated facilitation of virion release takes place at the plasma membrane. Also, facilitation of virion release is efficiently inhibited when Vpu is retained in the ER, suggesting that these two functions occur in two separate cellular compartments (51). Phosphorylation of two seryl residues (amino acids 52 and 56) by casein kinase II has been reported to affect Vpu functional phenotypes (13, 50). While phosphorylation status only partially affected the release function, it was necessary for efficient degradation of CD4, suggesting that Vpu phosphorylation status is likely to constitute an important control mechanism (51).

From a mechanistic standpoint, it is interesting that Vpu expression facilitates the release of divergent retroviruses, suggesting that its function is not specific to HIV (18). Vpu has structural and biochemical similarities with the influenza virus M2 protein, which has been suggested to exist as a multimeric ion channel capable of affecting luminal conditions such as pH in the Golgi compartment (32, 56). Similarly, Vpu has been demonstrated to be a type 1 integral membrane protein capable of homo-oligomerization (40). Whether some phenotypes associated with Vpu, similar to the influenza virus M2, are due to ion-channel effects awaits further exploration.

Nef

Although Nef, like Tat and Rev, is expressed early in the viral replication, as with other accessory proteins, its function is not essential in vitro. The phenotype associated with Nef in vitro is a matter of some contention. The controversy centers around the effect of Nef on viral gene expression. Initial studies which were instrumental in designating the protein Nef (for negative factor) characterized it as a down regulator of viral gene expression, possibly acting through long terminal repeat-directed transcriptional inhibition (1, 44). There have been contradictory reports, including studies that found no Nef-mediated transcriptional inhibition of HIV-1 replication (21) and kinetic studies which found a positive effect of Nef, with Nef mutant proviruses lagging behind their wild-type counterparts in various transformed and primary mononuclear cells (10, 31, 42, 58, 68). However, Nef sequences are highly polymorphic, and the functional variation associated with specific Nef sequences has been documented (52, 58, 68). Hence, some of the controversies over Nef function may be attributed to sequence variability and the problem of determining what constitutes the functional Nef.

Nevertheless, a well-established in vitro phenotype associated with Nef is the specific down regulation of surface CD4, a redundant function shared with Vpu (15, 16). However, while Vpu degrades CD4 in the ER, Nef appears to mediate endocytosis and lysosomal degradation of CD4 (2). As with Vpu, Nef-responsive sequences also lie in the cytoplasmic domain of CD4, and the membrane association of both Vpu (an integral membrane protein) and Nef (via myristylation) appears to be essential for their effects on CD4 (2, 5, 15, 35, 40, 60). Though it is likely that both these proteins may directly interact with CD4, to date no study has documented such an interaction.

Though an earlier study characterized Nef as a Ras-like G protein with both GTP-binding and GTPase activities, this has not been supported by subsequent reports (3, 20, 23). However, a cellular protein with serine kinase activity that associates with Nef has been identified, and Nef has been reported to interact with cytosolic and membrane proteins or cytoskeletal elements in a myristylation-dependent manner (22, 43, 49). Hence, most existing studies indicate that Nef's functional mechanism may involve interaction with cellular pathways possibly controlled by the differential biochemical modification status of Nef. However, exactly how these interactions affect viral replication remains to be investigated. In contrast to the controversial in vitro studies, some consensus regarding Nef's role in vivo has emerged in recent years. Although extensive sequence variability exists in Nef, the ORF itself is frequently retained in the HIV strains isolated to date (47). Importantly, Kestler et al. (30) found that SIV Nef mutants quickly reverted to functional ORFs in infected monkeys, suggesting a selective drive to foster intact Nef ORFs in vivo. Furthermore, monkeys that were unable to revert because of a deletion in Nef did not progress to clinical disease.

CONCLUSION

Several lines of evidence suggest that the accessory proteins encoded by HIV play an important role in the viral life cycle and disease pathogenesis: accessory protein reading frames are highly conserved among HIV strains and usually among distant lentivirus relatives, a finding indicative of a selective pressure to foster these ORFs. While it is clear that, unlike the regulatory proteins Tat and Rev, other auxiliary proteins are not required for productive viral replication in vitro, several independent studies have shown that optimal viral replication, even in vitro, requires expression of these accessory proteins, either generally or in a cell-specific manner. Viruses carrying mutations in one or more of these genes are severely impaired in cell types, such as macrophages, that may play a pivotal role in viral pathogenesis (4, 25, 62). While it is likely that the phenotypes observed in vitro are operational in vivo, it is possible that these proteins may have thus far unrecognized, and possibly indispensable, functions in vivo, not ascertainable by restrictive in vitro assays that have been employed to study them.

Importantly, emerging consensus suggests that these accessory proteins may be essential, and not dispensable, in vivo. Nef expression was associated with the depletion of thymocytes during HIV infection of SCID-hu mice (28). Similarly, Nef expression in transgenic mice resulted in perturbation of $CD4^+$ T-cell development and their depletion in peripheral blood (37, 52). Functional impairment of Vpr and Nef has been shown to retard disease progression in SIV-infected monkeys

(30, 33). Taken together, the results from these studies strongly suggest that some of the key events in HIV pathogenesis and disease progression may be associated with the expression of these accessory proteins. If the in vivo importance of these genes is corroborated by further evidence, it would be more appropriate to refer to these proteins as compensatory or virulence factors than as accessory proteins.

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