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MINIREVIEW

The Tale of the Long Tail: the Cytoplasmic Domain of HIV-1 gp41

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Envelope glycoproteins (Env) of lentiviruses typically possess unusually long cytoplasmic domains, often 150 amino acids or longer. It is becoming increasingly clear that these sequences contribute a diverse array of functional activities to the life cycle of their viruses. The cytoplasmic domain of gp41 (gp41CD) is required for replication of human immunodeficiency virus type 1 (HIV-1) in most but not all cell types, whereas it is largely dispensable for replication of simian immunodeficiency virus (SIV). Functionally, gp41CD has been shown to regulate rapid clathrin-mediated endocytosis of Env. The resultant low levels of Env expression at the cell surface likely serve as an immune avoidance mechanism to limit accessibility to the humoral immune response. Intracellular trafficking of Env is also regulated by gp41CD through interactions with a variety of cellular proteins. Furthermore, gp41CD has been implicated in the incorporation of Env into virions through an interaction with the virally encoded matrix protein. Most recently, the gp41CDs of HIV-1 and SIV were shown to activate the key cellular-transcription factor NF-κB via the serine/threonine kinase TAK1. Less well understood are the cytotoxicity- and apoptosis-inducing activities of gp41CD as well as potential roles in modulating the actin cytoskeleton and overcoming host cell restrictions. In this review, we summarize what is currently known about the cytoplasmic domains of HIV-1 and SIV and attempt to integrate the wealth of information in terms of defined functional activities.

BACKGROUND: BIOSYNTHESIS AND PROCESSING OF ENVELOPE

The target cell tropism of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) is determined by the virally encoded envelope glycoprotein (Env). Env consists of two components, the surface subunit (SU) gp120 and the transmembrane subunit (TM) gp41, which are produced from the precursor protein gp160 (1–3). In the plasma membranes of virions and infected cells, the mature Env complex is a trimer formed by three pairs of gp120 and gp41 (4–12). gp120, which is located entirely extracellularly, mediates sequential recognition of and binding to CD4 and the coreceptor (13–15); gp41 anchors the Env complex in the plasma membrane and is responsible for the fusion of viral and target cell membranes (16–18). The standard model of Env topology describes gp41 as a type I transmembrane protein, with one extracellular domain, one membrane-spanning domain, and one cytoplasmic domain (19, 20).

The precursor protein gp160 is translated from *env* mRNA at the rough endoplasmic reticulum (ER) and is modified cotranslationally by the addition of multiple N-linked carbohydrates (21–25) as well as O-linked carbohydrates (26, 27). While still within the ER, gp160 oligomerizes into homotrimers. Subsequently, trimeric gp160 is transported to the Golgi apparatus, where it is cleaved proteolytically by cellular furin or a cellular furin-like protease into gp120 and gp41 (28–32). gp120 and gp41 remain noncovalently associated after the cleavage (18), thus forming the mature trimeric Env complex. These Env complexes are then transported to the plasma membrane where they can be incorporated into budding virions (33, 34). Env trimers can be observed as "spikes" on the surface of virus particles by electron microscopy (12, 35, 36).

COMPARISON OF THE CYTOPLASMIC DOMAINS OF RETROVIRAL ENVELOPE PROTEINS

Arguably the most unusual feature of the lentiviral Env protein is the extraordinary length of its cytoplasmic domain. Env of most lentiviruses has a cytoplasmic domain longer than 130 amino acids (aa), the only exceptions being the closely related feline immunodeficiency virus (FIV; 50 aa) and puma lentivirus (PLV; 76 aa). In contrast, the analogous region of viruses from other retrovirus genera is typically shorter than 60 aa (Fig. 1).

The genomes of lentiviruses are highly adaptable; genetic information that conveys no advantage for viral fitness is lost quickly (39, 40). This implies that the unusually long cytoplasmic domain of lentiviruses is not a mere evolutionary artifact but rather contributes functional activities important for the virus. Indeed, extensive scientific investigation has identified a considerable variety of cellular interaction partners and functional activities of the cytoplasmic domain of HIV and SIV gp41 (gp41CD).

STRUCTURAL FEATURES AND TOPOLOGY

gp41CD can be divided into distinct domains based on biophysical properties (Fig. 2): the membrane-spanning domain is followed by a region of high hydrophilicity (20), which contains a highly immunogenic region (HIR) that frequently elicits high levels of antibodies in HIV-infected individuals and SIV-infected rhesus macaques (41–46). The C-terminal half of gp41CD contains two amphipathic segments believed to form α-helices, designated lentivirus lytic peptide 2 (LLP-2) and LLP-1 (47, 48). Additionally, a leucine zipper motif located between LLP-2 and LLP-1 has been identified and designated LLP-3 (49).

The high prevalence of antibodies against the HIR in sera from

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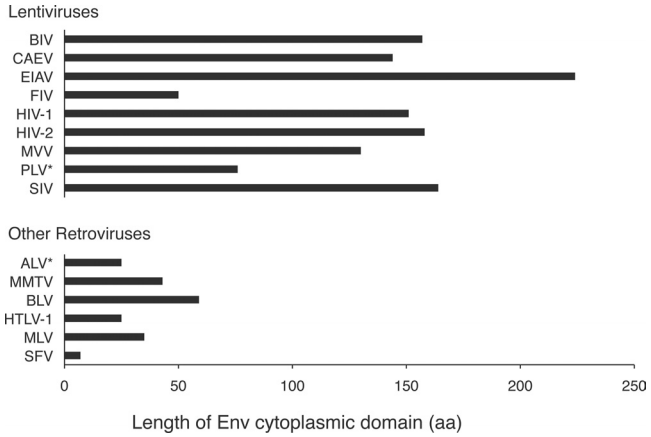

FIG 1 Comparison of Env cytoplasmic-domain lengths in lentiviruses and other retroviruses. Cytoplasmic-domain lengths are based on the UniProt database. BIV, bovine immunodeficiency virus; CAEV, caprine arthritis encephalitis virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; MVV, maedi/visna virus; PLV, puma lentivirus; SIV, simian immunodeficiency virus; ALV, avian leukosis virus (genus, *Alpharetrovirus*); MMTV, mouse mammary tumor virus (genus, *Betaretrovirus*); BLV, bovine leukemia virus (genus, *Deltaretrovirus*); HTLV-1, human T-lymphotropic virus 1 (genus, *Deltaretrovirus*); MLV, murine leukemia virus (genus, *Gammaretrovirus*); SFV, simian foamy virus (genus, *Spumavirus*). *, Prediction of cytoplasmic-domain length for ALV and PLV was not included in UniProt and was obtained with TMHMM 2.0 software (http://www.cbs.dtu.dk/services/TMHMM) (37). (Figure inspired by reference 38).

HIV-infected individuals has led to the proposal of an alternative model for gp41 topology in which sequences C-terminal to the membrane-spanning domain are not entirely contained within the cell or virion but instead form an extracellular loop which exposes the HIR to the extracellular medium (50–53). Recent studies have provided substantial evidence against this alternative model for Env of both HIV-1 and SIV (45, 54). While there is little proof to convincingly support the alternate topology, a transient (52) or low-level existence of such a structure cannot be rigorously excluded at this time.

Synthetic peptides corresponding in sequence to LLP-1, -2, and -3 interact with lipid bilayers *in vitro* (49, 55, 56), and gp41CD as a whole has been shown to weakly associate with lipid bilayers (19, 57). Based on this indirect evidence, a model has been proposed in which the LLPs form an association with the intracellular or intravirionic leaflet of the membrane (interaction partners of gp41CD are summarized in Table 1), with the hydrophobic face of each LLP buried in the membrane, while the hydrophilic faces are exposed to the cytoplasm (49, 55, 80). This model was recently corroborated by a study using iodonaphthyl azide labeling to detect membrane immersion of gp41CD (83).

It has been suggested that gp41CD may help to stabilize the Env trimer in its prefusion "spring-loaded" conformation, as truncation of gp41CD has been documented to enhance the fusogenicity of surface-expressed Env (84–89). LLP-2 appears to play a particularly important role for this stabilization (90, 91). The precise mechanism by which gp41CD consolidates the metastable prefusion state of Env is unknown, but it has been proposed to be due to (i) an interaction with the matrix (MA) domain of the viral Gag precursor protein (see below) (92–94) and/or (ii) a stabilizing effect of the association of gp41CD with the plasma membrane (91).

REQUIREMENT OF THE CYTOPLASMIC DOMAIN OF GP41 FOR REPLICATION

One of the most enigmatic phenomena regarding gp41CD is its cell-type-dependent requirement for HIV-1 replication. Truncation of gp41CD blocks replication of HIV-1 in most cell types, including peripheral-blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM). However, there are at least two cell lines in which HIV-1 with truncated Env is able to establish productive infection (Table 2). Truncation does not prevent proper processing of Env, nor does it inhibit the fusion process (88, 96–98, 100). When HIV-1 virions with truncated Env are produced in a permissive cell line, their single-round infectivity in both permissive and nonpermissive cells is almost as efficient as the single-round infectivity of wild-type virions (96, 97), implying that there is no structural defect associated with Env truncation that prevents entry into nonpermissive cell lines. However, cell types that are not permissive for replication of HIV-1 with truncated Env show a marked decrease in incorporation of truncated Env into virions, whereas permissive cell lines incorporate truncated Env at only slightly reduced levels into nascent virions (88, 96–100). This selective incorporation defect is likely the underlying cause for the cell type dependence of replication of HIV-1 with truncated Env. It is unknown why some cell lines incorporate truncated Env much more efficiently than others, at least in part because the process of Env incorporation itself is still incompletely understood (33). Regardless of the precise mechanism, it seems clear that the cytoplasmic domain of HIV-1 gp41 is critical for orchestrating the incorporation of Env into virions in most cell types.

There are only three known instances in which HIV-1 evolved truncated gp41CD variants. In each case, these adaptations were the result of extensive passaging in tissue culture and were accompanied by major compensatory changes (101–104). Conversely, SIV routinely evolves truncations of gp41CD when passaged in human cells, including cells that are not permissive for replication of HIV-1 with truncated Env (105–108). Indeed, it has been shown that truncation of SIV gp41CD increases Env incorporation into virions and consequently increases the levels of infectivity and fusogenicity (89, 109, 110). This offers a satisfying explanation for the phenomenon that SIV with truncated Env replicates more efficiently in human cells and thus quickly replaces viral species encoding full-length Env in culture (105–108). Intriguingly, however, truncation of Env appears to confer this selective advantage only in human cells, as gp41CD truncations do not evolve when SIV is cultured in rhesus PBMC (106–108). Consistent with this, SIV variants with a premature stop codon in gp41CD replicate less efficiently in rhesus PBMC than parental virus with full-length Env (107, 111), and reversion of gp41CD-truncated mutants to a full-length phenotype has been documented after prolonged passage in rhesus PBMC (106–108). It remains unknown why the selective advantage of SIV gp41CD truncations observed in human cells does not apply to SIV replication in rhesus-derived cells. As there are only very few rhesus cell lines, most tissue culture experiments examining the effects of SIV gp41CD truncation have been performed with human cell lines. Consequently, no information is available about any resultant influences of SIV gp41CD truncation on Env incorporation, infectivity, or fusogenicity in rhesus cells. A possible explanation for the negative effect of SIV gp41CD truncation on replication in rhesus,

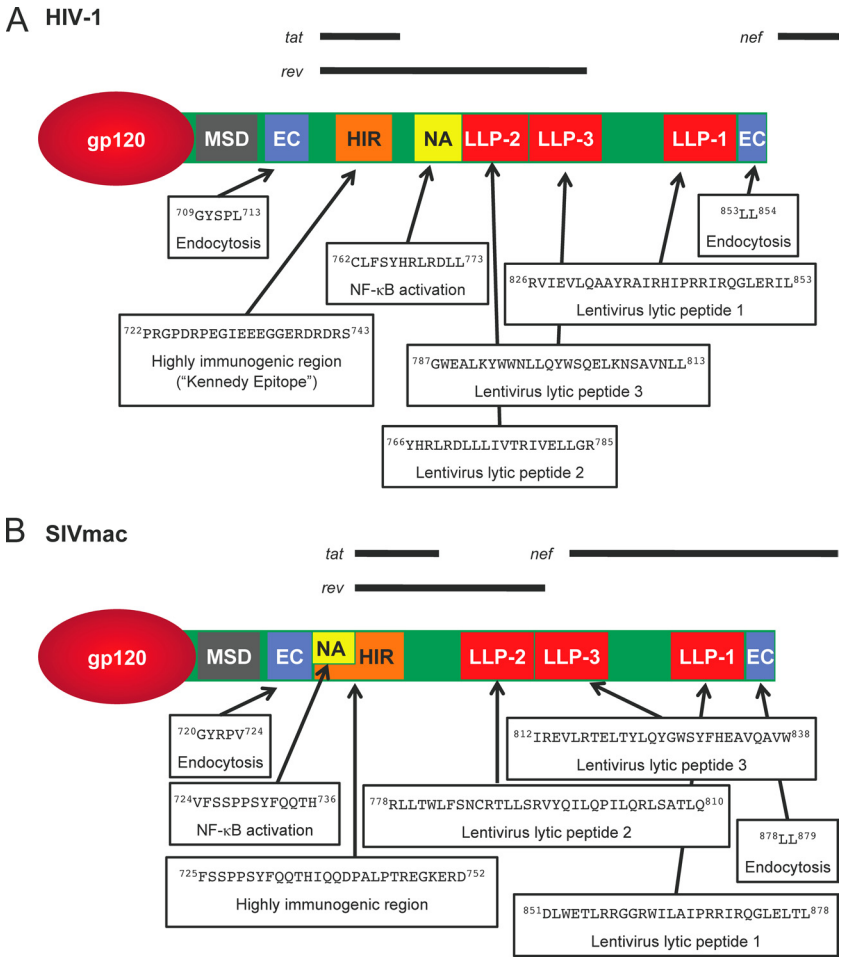

FIG 2 Schematic representation of gp41CD regions. (A) HIV-1. Amino acid sequences and numbering are based on full-length Env of strain NL4-3. (B) SIVmac. Amino acid sequences and numbering are based on full-length Env of strain SIVmac239. Black lines indicate the positions of overlapping open reading frames (ORFs) of *nef* and the second exons of *tat* and *rev* in the proviral genome. Note that LLP-2 and LLP-3 sequences of SIVmac are derived from sequence alignment with HIV-1 only, as there are to our knowledge no studies defining the SIV sequences for these regions. MSD, membrane-spanning domain; EC, endocytosis; HIR, highly immunogenic region; NA, NF-κB activation; LLP, lentivirus lytic peptide. See the text for details.

but not human, PBMC could be the presence of a hitherto-unrecognized host restriction factor. Such a restriction factor might inhibit the incorporation of SIV Env but be counteracted by full-length gp41CD. The human orthologue of this factor may not be effective against SIV, allowing SIV with truncated gp41CD to replicate freely in human cell lines, even in those not permissive for replication of HIV-1 with truncated gp41CD. The existence of this hypothetical factor would also offer an explanation for the selective incorporation defect of truncated HIV-1 Env discussed above. Indeed, the phenotype of this incorporation defect bears the hallmarks of a species-specific, differentially expressed restriction factor. In human cells permissive for replication of HIV-1 with truncated gp41CD, expression levels of this factor may be significantly lower than in nonpermissive cells. If such a putative restriction factor does exist, comparing the expression profiles of permissive and nonpermissive cell lines might provide a means to identify this factor.

Consistent with the observation that SIV with truncated gp41CD shows reduced viral fitness in rhesus PBMC during tissue culture experiments, gp41CD-truncated SIV mutants quickly revert to a full-length Env phenotype during *in vivo* infection of rhesus macaques (106, 108, 111). Moreover, a cohort of rhesus macaques infected with a rhesus macaque SIV (SIVmac) mutant that had gp41CD truncated through multiple redundant mutations which prevented reversion to a full-length phenotype, had markedly reduced viral loads and did not show clinical signs of progression to AIDS (111). Different selective pressures, such as the ability of the virus to evade the host immune response, are likely to influence the sequence evolution of gp41CD during infection *in vivo*. As discussed in further detail in the following section, HIV and SIV limit the levels of Env on the surface of cells and virions through gp41CD; this could be another selective force working against Env truncation during infection of rhesus macaques.

ENDOCYTOSIS

After transport to the cell surface, Env trimers of HIV-1 and SIV are rapidly internalized by clathrin-mediated endocytosis, resulting in low steady-state levels of Env surface expression (60, 64, 65, 112). This process is regulated by at least two separate domains of gp41CD.

The first established endocytosis motif conforms to the con-

TABLE 1 Interaction partners of HIV-1 gp41CD[a]

Interaction partner	Function	Amino acids of gp41CD required for binding	Conserved in SIV gp41CD	References
AP-1	Intracellular trafficking between TGN and endosome	709–713; 853–854	Yes	58, 59, 60, 61
AP-2	Clathrin-mediated endocytosis	709–713; 853–854	Yes	58, 59, 60, 62, 63, 64, 65
CaM	Apoptosis (?)	769–788 (LLP-2); 826–854 (LLP-1)	Yes	66, 67, 68, 69, 70, 71
α-Catenin	Reorganization of cytoskeleton (?)	787–813 (LLP-3)	Yes (?)	72, 73
Luman	Counteracting antiviral activity of luman (?)	749–766	Yes (?)	74
MA	Env incorporation	788–854 (LLP-3 and LLP-1)	Yes	75, 76
p115-RhoGEF	Disruption of RhoA signaling (?); reorganization of cytoskeleton (?)	769–854 (LLP-2, LLP-3, and LLP-1)	No	38, 77
Perilipin-3	Intracellular trafficking; MA interaction (?); Env incorporation (?)	784–822 (LLP-3)	ND	76, 78, 79
Plasma membrane (?)	Stabilization of Env in prefusion conformation (?)	769–788 (LLP-2); 787–813 (LLP-3); 826–854 (LLP-1)	Yes (?)	19, 49, 56, 57, 80
PRA1	Intracellular trafficking (?)	Unknown for HIV-1; 776–792 for SIV (LLP-2)	Yes	38
Prohibitin 1/2	Overcoming block in nonpermissive cells (?)	788–798 (part of LLP-3)	ND	81
TAK1	NF–κB activation	762–773	No	82

[a] Amino acid (aa) numbering is based on the sequence of the full-length NL4-3 envelope (GenBank accession no. AF324493.2). TGN, *trans*-Golgi network; (?), interaction/function not fully established; ND, not determined.

sensus sequence YXXΦ (where X is any and Φ a hydrophobic amino acid) and is located close to the membrane-spanning domain (Fig. 2, amino acids ^{709}GYSPL^{713} in HIV-1 NL4-3 and ^{720}GYRPV^{724} in SIVmac239) (64, 65, 112). The cellular interaction partner mediating endocytosis through this membrane-proximal YXXΦ motif is the μ2 subunit of the clathrin-associated adaptor protein complex AP-2 (Table 1) (58–60, 62). Any effects resulting from mutation of this YXXΦ motif on viral infectivity and fusogenicity in tissue culture-based experiments may vary with the virus strain, the cell type, and the nature of the assays used (58, 110, 113–115). However, disruption of this motif in SIV gp41CD results in markedly lower viral loads and strongly reduced pathogenic potential during infection of rhesus macaques (116).

TABLE 2 Summary of cell lines tested for permissiveness of HIV-1 replication and incorporation of Env with truncated gp41CD[a]

Cell line	Description	Replication	Incorporation	References
MT-4	Human CD4$^+$ T-cell line isolated from patient with T-cell leukemia; HTLV-1 transformed but not HTLV-1 producing	++	++	88, 95, 96
M8166	Human CD4$^+$ T-lymphoblastoid cell line derived from C8166; HTLV-1 transformed but not HTLV-1 producing	++	+++	97
H9	Human CD4$^+$ T-cell line derived from HUT 78 cells, which were isolated from patient with Sézary syndrome	—	+	97, 98, 99
CEM (12D7)	Human CD4$^+$ T-lymphoblastoid cell line derived from A3.01, which is derived from CEM, a cell line established from blood of a patient with acute lymphoblastic leukemia	—	—	96
Jurkat	Human CD4$^+$ T-lymphoblastoid cell line, established from blood of patient with acute T-cell leukemia	—	—	96
huMDM	Human monocyte-derived macrophages	—	—	96
huPBMC	Human peripheral-blood mononuclear cells	—	ND	96, 99
CEMx174	Somatic-cell hybrid line created by fusion of human B-cell line 174 and CEM cells	—	ND	98
SupT1	Isolated from patient with non-Hodgkin's T-cell lymphoma; expresses CD4 at high levels	—	ND	99
MT-2	Human CD4$^+$ T-cell line isolated from patient with T-cell leukemia; HTLV-1 transformed and HTLV-1 producing	—	ND	96, 99
CV-1	RSV-transformed kidney epithelium cells from African green monkey (*Chlorocebus sabaeus*)	N/A	++	88
HeLa	Human cell line derived from patient with cervical epithelial carcinoma	N/A	++	96
HEK293T	Human embryonic kidney cell line originally created by transformation with DNA from adenovirus type 5; expresses SV40 large T antigen	N/A	+++	92

[a] Env with truncated gp41CD has a deletion of at least 144 amino acids. RSV, Rous sarcoma virus; SV40, simian virus 40; ND, not determined; N/A, not applicable (replication assays not possible because cells do not express CD4); +++, equal to that of wild type; ++, slightly less than that of wild type; +, markedly less than that of wild type; —, below threshold of detection.

The membrane-proximal YXXΦ motif is extremely well conserved among isolates of HIV-1 groups M, N, and O and of chimpanzee SIV (SIVcpz) (present in 175 of 176 sequences examined), as well as of HIV-2, SIVmac, pigtailed macaque SIV (SIVmne), sooty mangabey SIV (SIVsmm), and stump-tailed macaque SIV (SIVstm) (68 of 68 sequences) (117). gp41CD of HIV-1 has a second, similarly well-conserved YXXΦ motif located closer to the C terminus of HIV-1 gp41CD (172 of 176 sequences; amino acids ^{766}YHRL^{769} in HIV-1 NL4-3) (117), which does not mediate significant endocytosis but induces NF-κB activation (see below) (62, 65, 82). SIV gp41CD features two additional YXXΦ motifs, one highly conserved in HIV-2, SIVmac, SIVmne, SIVsmm, and SIVstm (68 of 68 sequences; amino acids ^{768}YIHF^{771} in SIVmac239), the other conserved only within SIVmac, SIVmne, SIVsmm, and SIVstm (40 of 44 sequences; amino acids ^{795}YQIL^{798} in SIVmac239) and not at all in HIV-2. Neither of these secondary YXXΦ motifs mediates detectable levels of endocytosis (59).

The second functional endocytosis motif identified in HIV-1 gp41CD is a dileucine motif at the very C terminus (Fig. 2), which also utilizes the AP-2 complex for internalization (63). Disruption of this dileucine motif by itself only leads to a small increase in cell surface expression of Env but results in a strong synergistic effect when mutated in combination with the membrane-proximal YXXΦ motif (63). Typically, endocytosis-mediating dileucine motifs recruit the σ2 subunit of the AP-2 complex (118), but such an interaction between gp41CD and σ2 has yet to be formally demonstrated. Surprisingly, this dileucine motif (amino acids ^{853}LL^{854} of sequence ^{849}LERILL^{854} in HIV-1 NL4-3) does not fully conform to the consensus dileucine motif for clathrin-mediated endocytosis ([D/E]XXXL[L/I/M]) (118), and it is not pervasively conserved within HIV-1 and SIVcpz (135 of 176 sequences) (117). There is strong evidence that gp41CD of SIV also contains a second endocytosis motif close to the C terminus, as truncations C-terminal to the membrane-proximal YXXΦ motif have been shown to increase cell surface expression of SIV Env, and disruption of the membrane-proximal YXXΦ motif reduces Env endocytosis more strongly when part of gp41CD is removed (58, 59, 110). Consistent with this observation, the C-terminal dileucine motif of HIV-1 gp41CD is highly conserved in gp41CD of HIV-2, SIVmac, SIVmne, SIVsmm, and SIVstm (67 of 68 sequences; amino acids ^{878}LL^{879} in SIVmac239) (117) (Fig. 2).

In addition to the C-terminal L[L/I] motif, gp41CDs of HIV-1 and SIV have multiple L[L/I] motifs (six in HIV-1, four in SIVmac), with various degrees of conservation among isolates. None of these motifs conform to the consensus motif for endocytosis [D/E]XXXL[L/I/M]. Studies in which these additional endocytosis motifs were mutated did not detect major effects on cell surface expression of Env (59, 113). Nonetheless, there is some evidence that the gp41CD, at least of SIV, contains additional signals that contribute to endocytosis (59). It is not clear whether residual endocytosis in the absence of the established endocytosis motifs of gp41CD is mediated by signals that do not conform to the known consensus motifs or whether multiple redundant endocytosis motifs mask the contribution of individually mutated motifs. Further studies will be required to comprehensively elucidate the complexities of Env endocytosis.

Interestingly, Egan et al. reported that the presence of HIV-1 Gag was sufficient to counteract the endocytosis of Env, presumably because binding of the Gag domain matrix (MA) to gp41CD (see following section) blocks the interaction of gp41CD with AP-2 (119). In order to be efficiently incorporated into the virion, sufficient numbers of Env molecules need to be present on the cell surface, and consequently it has been suggested that Gag prevents endocytosis of Env molecules that are about to be incorporated, while redundant Env is removed from the cell surface (119). Contrary to this, it has been demonstrated that CEMx174 cells chronically infected with SIVmac239 rapidly endocytose SIV Env from their surfaces despite the presence of SIV Gag (59, 64). This disparity could possibly reflect differences between HIV-1 and SIV in the interactions of their respective Env and Gag proteins. To fully understand the relevance of these observations, further studies will be needed.

The rapid endocytosis of Env from the cell surface translates into a low number of Env trimers incorporated per virion. The number of spikes per virion has been estimated to range from 7 to 16, as calculated based on the molar ratio of Env to Gag by high-performance liquid chromatography (HPLC) (120) and Western blotting (110), as well as visual detection by electron tomography (12). All of these different methods performed by independent laboratories yielded remarkably consistent results. The same studies also found that SIV variants with truncated gp41CD invariably show much higher levels of Env incorporation, ranging from ca. 10- to 40-fold more Env trimers per virion than SIV with full-length gp41CD. Yuste et al. compared several SIV gp41CD mutants with premature stop codons or a mutation of the membrane-proximal YXXΦ motif and observed that the level of Env surface expression was closely mirrored by the level of Env incorporation into virions (110).

Why would HIV-1 and SIV evolve mechanisms to minimize the level of Env on the surfaces of host cells and virions? The most likely explanation is that it serves as a mechanism for immune evasion. The presence of Env on the cell or virion surface exposes viral epitopes to antibody binding, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-mediated cytotoxicity. Consequently, limiting the amount of Env on the cell surface could reasonably provide HIV and SIV with some degree of protection against the humoral branch of the immune system, although this protection must necessarily be incomplete to allow for Env incorporation into virions and viral entry. This theory is supported by the observation that a hard-to-revert mutation which increased the cell surface expression of SIV Env by disrupting the membrane-proximal endocytosis motif of gp41CD resulted in severely attenuated pathogenicity of SIV infection in rhesus macaques, consistent with better control of viral infection by the host's immune system (116).

ENVELOPE INCORPORATION INTO VIRIONS

The matrix (MA) component of the Gag precursor protein has long been known to be an essential regulator of Env incorporation into HIV and SIV virions (115, 121–124). MA tethers the Gag polyprotein to the inner leaflet of the membrane through a myristoylated, membrane-binding domain (125–129). Targeting of Gag to the budding site at the plasma membrane is also directed by MA (130–133) and involves direct binding of MA to phosphatidylinositol (4,5)bisphosphate on the inner leaflet (136–139). At the membrane, MA and Env interact through gp41CD, but the exact nature of this interaction is not entirely clear (Table 1). A small deletion in the LLP-2/LLP-3 region of gp41CD that blocks Env incorporation into HIV-1 virions was shown to be counteracted by mutations in MA (140); conversely, substitutions of sin-

gle amino acids in MA can block incorporation of Env into virions (95, 141). Surprisingly, mutations in MA that block Env incorporation can be overcome by truncation of the gp41CD N-terminal of LLP-2 (85, 95, 141, 142). These data suggest that an interaction between Env and MA may not be required for Env incorporation *per se* but may be necessary to structurally accommodate the presence of the long cytoplasmic domain of gp41. In this context, it is interesting to note that MA, like gp41, may assemble into trimers or hexamers of trimers (126, 143), although the definitive *in vitro* structure is not yet known. Alternatively, the higher levels of Env at the cell surface resulting from truncation of gp41CD (see above) may simply allow for sufficient Env to become incorporated into virions despite the MA mutation.

How exactly gp41CD and MA interact physically is controversial. An early publication by Cosson reported a direct interaction of purified, bacterially expressed Env and MA that mapped to the C-terminal half of gp41CD (75). In direct contradiction to these findings, Lopez-Vergès et al. later described the intracellular trafficking protein perilipin-3, more commonly known as tail-interacting protein of 47 kDa (TIP47), as an essential connector between gp41CD and MA which mediates their interaction as part of a heterotrimeric complex that is indispensable for Env incorporation into HIV-1 virions in $CD4^+$ T cells and HeLa cells (Table 1) (79). The same group recently reported similar findings for HIV-1 infection of primary macrophages (144). A different study confirmed the interaction between perilipin-3 and HIV-1 gp41CD, but the presence or absence of perilipin-3 did not seem to affect *in vitro* binding of gp41CD to MA (76). The same publication also demonstrated a direct interaction between SIV MA and SIV gp41CD and, interestingly, between SIV MA and HIV-1 gp41CD (76). Further studies are needed to resolve the specifics of gp41CD/MA interaction. Perilipin-3 has also been suggested to be involved in intracellular trafficking of Env, as described in more detail below.

INTRACELLULAR TRAFFICKING

gp41CD regulates intracellular trafficking of Env through a variety of interaction partners. The membrane-proximal YXXΦ motif in the gp41CDs of HIV-1 and SIV and the C-terminal dileucine motif of HIV-1, which mediate endocytosis (see above), have been shown to bind the μ1 subunit of the AP-1 complex in addition to μ2 of the AP-2 complex (Table 1) (58–62, 65). Unlike the AP-2 complex, whose principal function is clathrin-mediated endocytosis from the plasma membrane, the AP-1 complex is primarily involved in clathrin-mediated trafficking between the *trans*-Golgi network (TGN) and endosomes (145). In polarized epithelial cells, the AP-1 complex is able to effect targeting to the basolateral membrane (145). This last property is consistent with the observation that the membrane-proximal YXXΦ motif of gp41CD directs the location of HIV-1 budding in Madin-Darby canine kidney (MDCK) cells (146) and Jurkat T cells (147). In the case of Jurkat T cells, this polarized budding was shown to be relevant for cell-to-cell viral transmission (147). Specifically, AP-1-mediated trafficking of Env might play an important role in targeting Env to the virological synapse during T-cell-to-T-cell transmission (148).

Another interaction partner of gp41CD that is involved in intracellular trafficking is the cellular protein perilipin-3 (Table 1) (78). The interaction between gp41CD and perilipin-3 requires the presence of a diaromatic YW motif contained within the LLP-3 region (^{800}YW^{801} in Env of HIV-1 NL4-3). Perilipin-3 has been reported to be essential for the retrograde transport of mannose-6-phosphate receptors from endosomes to the TGN (149). Interestingly, perilipin-3 is also a member of the PAT family (thus named for the member proteins perilipin, adipophilin, and TIP47) of lipid droplet-associated proteins and as such is involved in lipid metabolism (150). The physiological relevance of perilipin-3 binding to gp41CD is not entirely clear, but disruption of this association altered the subcellular localization of Env (78, 144). It is worth noting that a cellular interaction partner of perilipin-3, the GTPase Rab9 (151), was independently shown to be required for efficient replication of HIV-1 (152).

Interestingly, another intracellular-trafficking protein reported to interact with gp41CD of both HIV-1 and SIV, the prenylated Rab acceptor 1 (PRA1) (Table 1) (38), also interacts functionally with Rab9 (153). PRA1 was identified as an interaction partner of SIV gp41 in a yeast two-hybrid screen (38). Binding could not be verified by coimmunoprecipitation or glutathione *S*-transferase (GST) pulldown but was detectable with modest efficiency in a mammalian two-hybrid assay. Based on the same assay, an interaction of PRA1 with HIV-1 gp41CD was also identified. The interaction between SIV gp41CD and PRA1 mapped to the LLP-2 region (38). However, a subsequent study found that neither overexpression of PRA1 nor its downregulation by RNA interference (RNAi) had any measurable effect on Env incorporation, infectivity, or virus production (154). The observation that depletion of PRA1 had no effect on various aspects of the life cycle of HIV-1 and SIV raises the question of whether PRA1 is truly a relevant interaction partner of gp41CD.

In addition to these protein interactions, gp41CD harbors determinants that have been reported to target Env to detergent-resistant membrane fractions, also known as lipid rafts. A highly conserved cysteine located in the vicinity of the LLP-2 region of HIV-1 and SIV gp41CD is subject to palmitoylation, which might serve as a membrane anchor and stabilize the association of gp41CD with the plasma membrane (80). A second cysteine located closer to the C terminus has also been shown to be palmitoylated but is less conserved among HIV-1 isolates and is absent in SIV isolates (117). The palmitoylation of gp41CD cysteines (or their replacement with bulky hydrophobic amino acids) has been suggested to target Env to lipid rafts (155, 156). Bhattacharya et al. reported that targeting of Env to lipid rafts enhances Env incorporation into virions and infectivity but is not required for either (155). Chen and colleagues have questioned the role of palmitoylation and have suggested instead that the LLP regions are principally responsible for targeting of Env to lipid rafts (157, 158).

CELLULAR ACTIVATION

Recently, the gp41CDs of HIV-1 and SIV were shown to induce activation of NF-κB, providing the first description of a role for gp41CD in the regulation of cellular activation (82). NF-κB (for nuclear factor κB) describes a heterogeneous family of transcription factors that are essential regulators of diverse biological processes, including cellular proliferation, survival, and differentiation, as well as innate and adaptive immunity (159). Importantly, NF-κB also serves as a major regulator for the transcription of the HIV-1 and SIV proviral genomes. The long terminal repeat (LTR) region of HIV-1 typically has two NF-κB-binding sequences; clade C isolates have at least three (160–163). The LTR of SIVmac has one NF-κB-binding site (164). As a result of considerable re-

dundancy of *cis*-acting elements within the LTR, these NF-κB-binding sites are not absolutely required for replication of HIV-1 or SIV (165–168). However, activation of NF-κB can drastically enhance LTR-dependent transcription (161, 169, 170), and stimulation of HIV-1-infected cells with NF-κB-activating cytokines can strongly increase viral replication (134, 171, 172). gp41CD of HIV-1 and SIV induces NF-κB activation through the canonical pathway, and this process requires activation of the serine/threonine kinase transforming growth factor β (TGF-β)-activated kinase 1 (TAK1) (82). TAK1 is a central regulator of NF-κB activation upon which several branches of the canonical pathway converge. TAK1 phosphorylates and thereby activates the inhibitor of κB kinase (IKK) complex, which in turn phosphorylates inhibitor of κB (IκB) proteins and thus marks them for proteasomal degradation. Removal of IκB proteins allows cytoplasmic NF-κB dimers to translocate into the nucleus and act as regulators of transcription (173, 174). gp41CD of HIV-1 interacts physically with TAK1, and this interaction requires the same region of the cytoplasmic domain that is required for NF-κB activation (Fig. 2 and Table 1) (82), implying a causal relationship between TAK1 binding and induction of NF-κB activation by HIV-1 gp41CD. This region of HIV-1 gp41CD partly overlaps with LLP-2 and includes the second, distal YXXΦ motif (see above). SIV gp41CD does not interact detectably with TAK1 but appears to intersect the canonical NF-κB pathway through a signaling protein upstream of TAK1. This signaling protein remains to be identified. A mutant of HIV-1 NL4-3 deficient for gp41CD-mediated NF-κB activation did not display impaired infectivity or replication in fully activated cells but was unable to replicate efficiently in suboptimally activated host cells in which wild-type virus replicated successfully. Importantly, this defective phenotype of the gp41CD mutant could be rescued by exogenous stimulation of the host cells (82). These observations imply that the capacity of gp41CD to induce activation of NF-κB is important for the virus's ability to replicate under conditions of limited cellular activation and thus may be particularly relevant for replication *in vivo*, where the virus is likely to continuously encounter non- or suboptimally activated lymphocytes.

CYTOTOXICITY AND APOPTOSIS

Shortly after the isolation of HIV-1, it was suggested that gp41CD is responsible, at least in part, for the strong cytopathic effects observed during infection in cell culture (175, 176). Consistent with this notion, synthetic peptides corresponding in sequence to the LLP regions have the ability to lyse prokaryotic and eukaryotic cells *in vitro*, leading to their designation as lentivirus lytic peptides (55, 56, 177, 178). It is not clear what significance the cytotoxic potential of the individual LLP regions has in the context of the whole Env protein, much less during infection, and how much they contribute to the cytopathicity of infection by HIV or SIV. Costin et al. have suggested that the LLP regions might form a membrane pore in the context of the complete Env trimer and thus cause cytopathicity through uncontrolled ion flux between the cytosol and the extracellular medium (179, 180); however, there is currently limited evidence to support this hypothesis.

In addition to the cytopathic potential inherent in the LLP regions, the gp41CDs of HIV-1 and SIV have also been shown to interact with a regulator of apoptosis, calmodulin (CaM) (Table 1) (66–71). CaM is a major sensor and regulator of intracellular Ca^{2+} concentration and, in addition to apoptosis regulation (181), is involved in a plethora of different cellular processes, including lymphocyte activation via the transcription factor family NF-AT (182, 183). Synthetic peptides corresponding to LLP-1 and LLP-2 sequences of HIV-1 and SIV have been shown to interact with CaM (68–71), but binding in the context of full-length gp41CD appears to be confined largely to LLP-1, at least in the case of HIV-1 (66, 67). HIV-1 gp41CD induces an increase in the intracellular concentration of Ca^{2+}, leads to spontaneous apoptosis, and enhances FAS-mediated apoptosis; these effects can be blocked by CaM inhibitors and by mutations that abrogate the interaction between CaM and gp41CD (66, 67, 184–186). Mutant virus that is unable to bind to CaM through gp41CD was shown to replicate with kinetics indistinguishable from that of wild-type virus in tissue culture (184).

What benefit—if any—might the virus derive from inducing necrosis and/or apoptosis in the infected host cell? It is certainly conceivable that these cytotoxic effects are an unavoidable consequence of maximizing virus production and infectivity. Other possible explanations for the cytotoxicity ascribed to the LLP regions and the observed interaction between gp41CD and CaM include the following.

(i) The cytotoxicity of gp41CD might be exaggerated under conditions of experimental Env overexpression. Cytotoxic expression levels may not be reached during infection *in vivo*.

(ii) The cytotoxic sequences might have beneficial properties that outweigh any negative consequences of killing the host cell. For instance, the LLP regions are believed to form amphipathic helices, providing the biophysical basis for the postulated interaction of gp41CD with the membrane, which may be important for targeting of Env to lipid rafts and for stabilizing the Env trimer in the prefusion conformation (see above). The ability to interact with both hydrophobic and hydrophilic surfaces brings with it an inherent potential for membrane disruption, making it difficult to separate the ability to associate with the membrane from the cytotoxic potential. Similarly, CaM is a highly versatile regulatory protein that is involved in activities other than apoptosis (182, 183). It is possible that the interaction between gp41CD and CaM has evolved to exploit another function of CaM, for instance, its role in lymphocyte activation (182, 183). As optimal replication of HIV and SIV requires fully activated host cells (187–190), using CaM to enhance the state of cellular activation would offer an obvious advantage to viral replication that may outweigh the concomitant disadvantage of inducing apoptosis.

(iii) Env is expressed late in the viral life cycle. It is possible that Env does not accumulate to cytotoxic levels before the host cell has already released large amounts of viral progeny, at which point the death of the host cell may be of limited consequence to the virus.

INTERACTIONS WITH REGULATORS OF THE CYTOSKELETON

Virtually all known vertebrate viruses, including HIV, manipulate the actin cytoskeleton during their replication cycles. The cortical actin fibers form a densely polymerized meshwork adjacent to the cell membrane and constitute a considerable physical obstacle to a virus (191). Manipulation of the actin cytoskeleton may allow the virus to penetrate the cortical actin network upon entry and egress (192, 193). While direct manipulation of actin filaments by gp41CD has not been experimentally established, gp41CD has been shown to interact with at least two proteins that are involved in the regulation of the actin cytoskeleton.

Specifically, HIV-1 gp41CD interacts with p115-RhoGEF, a

guanine nucleotide exchange factor and activator of the RhoA GTPase (Table 1) (77). RhoA is a central regulator of actin stress fibers and focal adhesions (194) and is the target of manipulation by several other viruses (193). Additionally, RhoA is involved in the regulation of cell cycle progression and the transcription factors serum response factor (SRF) and NF-κB (195). Importantly, activation of RhoA by p115-RhoGEF has been shown to inhibit replication of HIV-1 (196). The mechanism of this inhibition has never been resolved, but it surprisingly appears to be unrelated to actin reorganization. Wang et al. were able to show that inhibition of HIV-1 transcription by RhoA activation was independent of the known RhoA functions of stress fiber formation, SRF activation, and transformation and therefore suggested RhoA might inhibit HIV-1 transcription through a novel, hitherto-unknown pathway (196). The interaction between gp41CD and p115-RhoGEF required the presence of LLP-2, LLP-3, and LLP-1 and could be abrogated by single-amino-acid substitutions in LLP-3, e.g., L798R (77). Intriguingly, the L798R mutation blocked viral replication in three different cell lines. Conversely, the presence of wild-type gp41CD inhibited p115-RhoGEF signaling, including RhoA activation (77). Although it was never formally demonstrated, it seems likely that the inhibition of p115-RhoGEF by gp41CD binding has evolved to inhibit activation of RhoA and thus to facilitate viral replication. gp41CD of SIV did not detectably interact with p115-RhoGEF (38).

Interestingly, RhoA interacts functionally and physically with the cadherin complex, which includes another interaction partner of gp41CD, α-catenin (Table 1) (72, 73, 197). α-Catenin is primarily known for its pivotal role in cell-cell adhesion as part of the α-catenin/β-catenin/cadherin complex. During the formation of intercellular cadherin interactions, α-catenin is believed to induce the reorganization of the actin cytoskeleton by binding to actin filaments (198). Recently, additional activities have been attributed to α-catenin, including a role in the regulation of cell proliferation and hedgehog signaling (199). Kim et al. reported that HIV-1 gp41CD and α-catenin interact directly through the LLP-3 region (72, 73). SIV gp41CD also interacted with α-catenin in yeast two-hybrid assays, but the group did not confirm this interaction with a more rigorous assay (72). The interaction with HIV-1 gp41CD mapped to the C-terminal region of α-catenin, which also harbors the actin-binding domain. β-Catenin did not interact directly with gp41CD (73). As cadherin complexes mediate cell-cell adhesion, it has been suggested that gp41CD binding to α-catenin might be involved in cell-to-cell transmission of HIV-1 (73). However, to our knowledge there is currently no report that implicates cadherins and/or α-catenin in the formation of cell contacts between T cells. Alternatively, α-catenin might serve as an adaptor between gp41CD and the actin cytoskeleton, serving either to target Env to the site of viral budding or to reorganize the actin cytoskeleton to facilitate egress. Attractive hypotheses notwithstanding, a physiological role for the interaction of α-catenin with gp41CD remains to be demonstrated.

Of note, α-catenin has been reported to interact with gp41CD in the same region as p115-RhoGEF, inviting speculation that the separately published interactions of α-catenin and p115-RhoGEF with gp41CD might indeed be related phenomena.

OVERCOMING CELLULAR RESTRICTION

Two recently identified interaction partners of gp41CD, luman (74) and prohibitin 1/2 (81) (Table 1), indicate that gp41CD might also act to overcome certain aspects of cellular restriction of HIV replication.

Luman (also known as CREB3 or LZIP) is a poorly characterized member of the OASIS family of transcription factors (200). Luman appears to be associated with the ER stress response, or unfolded protein response (UPR) (200). Both known target genes of luman are involved in ER-associated degradation (ERAD) of unfolded protein (200). Blot et al. found that the ER membrane-bound, inactive, full-length form of luman interacted with gp41CD of HIV-1, SIV, and HIV-2 in yeast two-hybrid assays (74). For HIV-1 gp41CD, this interaction was confirmed by co-precipitation and mapped to a region N-terminal of LLP-2. Expression of gp41CD led to accelerated degradation of full-length luman, implying that the interaction between gp41CD and luman has evolved to reduce levels of luman in the infected cell. Interestingly, a cleaved, constitutively active form of luman reduced proviral transcription (74). The mechanism by which activated luman represses proviral transcription and what role these interactions play during infection remain to be elucidated; however, gp41CD may have evolved to counteract the antiviral effects of luman.

Emerson et al. have identified an interaction between HIV-1 gp41CD and a (presumably heterodimeric) complex of the proteins prohibitin 1 and prohibitin 2 (81). Prohibitin 1/2 are involved in a multitude of cellular processes, including signal transduction, mitochondrial function, apoptosis, and cell proliferation (201, 202). They are localized primarily to mitochondria but have also been detected at the plasma membrane and in the nucleus (202). The interaction between gp41CD and prohibitin 1/2 mapped to a region in the center of LLP-3 (81). Mutations of gp41CD that abrogated binding to prohibitin 1/2 produced a replication phenotype reminiscent of complete gp41CD truncations; i.e., mutant HIV-1 was able to replicate in a cell line permissive for gp41CD truncation but not in a nonpermissive cell line (see above). This prompted the authors to speculate that prohibitin 1/2 binding by gp41CD may be essential for HIV-1 replication in nonpermissive cell lines. It is not clear how prohibitin 1/2 might influence viral replication, as Env incorporation into virions was not affected in the absence of prohibitin 1/2 binding to gp41CD, and no other mechanism has been proposed (81).

Serra-Moreno et al. recently described a particularly interesting example of gp41CD adaptation to counteract a cellular restriction factor (203). They reported that an SIV variant from a rhesus macaque that had been infected with a form of SIVmac with a *nef* deletion had acquired the ability to bind to and counteract the cellular restriction factor bone marrow stromal antigen 2 (BST-2; also known as CD317, HM1.24 antigen, or tetherin) via its gp41CD (203). BST-2 is an interferon-inducible factor that is able to inhibit the spread of enveloped viruses by "tethering" them to the cell surface after egress and mediating their endocytosis (204–206). HIV-1 typically uses its Vpu protein to bind to and counteract BST-2, while HIV-2 uses the extracellular domain of its Env protein. Most SIV strains counteract BST-2 through Nef (204). Infection of rhesus macaques with *nef*-deleted SIVmac (SIVmac Δ*nef*) typically has a considerably attenuated phenotype (207). However, the infection persists at low levels, and in some cases, the virus can acquire compensatory mutations that restore its pathogenic potential to various degrees (135, 208). Serra-Moreno et al. studied one such variant of SIVmac Δ*nef* with increased pathogenic potential and found that it had accumulated mutations in its

LLP-3 and LLP-1 regions of gp41CD that allowed it to bind to and counteract BST-2 (203). Interestingly, inhibition of BST-2 activity, but not binding to BST-2, required the membrane-proximal YXXΦ motif of gp41CD (203). This example illustrates the impressive functional versatility and adaptability of gp41CD.

CONCLUDING REMARKS

It now seems quite clear that the cytoplasmic domain of the HIV-1 and SIV gp41 glycoproteins contributes a variety of different functional activities to the viral life cycle. In particular, these include endocytosis to regulate levels of Env surface expression, incorporation of Env into virions, intracellular trafficking of Env, and activation of the host cell. Some of the cellular interaction partners mediating these functions have been reasonably well characterized. However, several other interaction partners of gp41CD whose functional relevance is less clear have been described. These interactions seem to implicate gp41CD in an even wider range of functions, including apoptosis induction, actin modulation, and countering aspects of cellular restriction. Assessment of the significance of these interaction partners is currently incomplete and will require further investigation. Considering that α-catenin, p115-RhoGEF, prohibitin 1/2, and perilipin-3 all have been reported to interact with gp41CD in the same region (Table 1), use of a mutational approach to gauge relative importance and phenotypic manifestations will be a daunting challenge.

In addition to these relatively new questions, the long-standing puzzle of the cell-type-dependent requirement of gp41CD for viral replication remains an exciting area of research. The complex phenotype observed with truncated gp41CD of HIV-1 and SIV bears the hallmarks of a species-specific and differentially expressed restriction factor that can be counteracted by gp41CD; however, whether such a restriction factor does indeed exist and, if so, the identity of this factor remain to be elucidated. Another intriguing question is whether difficult-to-revert mutations that reduce Env endocytosis result in more effective immunological control of viral replication. Answering this question will undoubtedly require creative experimental design using the rhesus macaque model of SIV infection.

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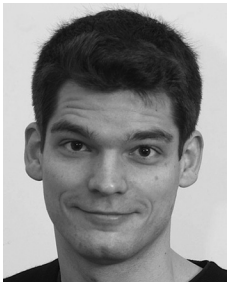

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