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J Mol Biol. Author manuscript; available in PMC 2012 July 22.

Published in final edited form as:
J Mol Biol. 2011 July 22; 410(4): 582–608. doi:10.1016/j.jmb.2011.04.042.

HIV-1 Envelope Glycoprotein Biosynthesis, Trafficking, and Incorporation

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Abstract

The HIV-1 envelope (Env) glycoproteins play an essential role in the virus replication cycle by mediating the fusion between viral and cellular membranes during the entry process. The Env glycoproteins are synthesized as a polyprotein precursor, gp160, that is cleaved by cellular proteases to the mature surface glycoprotein gp120 and the transmembrane glycoprotein gp41. During virus assembly the gp120/gp41 complex is incorporated as heterotrimeric spikes into the lipid bilayer of nascent virions. These gp120/gp41 complexes then initiate the infection process by binding receptor and co-receptor on the surface of target cells. Much is currently known about the HIV-1 Env glycoprotein trafficking pathway and the structure of gp120 and the extracellular domain of gp41. However, the mechanism by which the Env glycoprotein complex is incorporated into virus particles remains incompletely understood. Genetic data support a major role for the cytoplasmic tail of gp41 and the matrix domain of Gag in Env glycoprotein incorporation. Still to be defined are the identities of host cell factors that may promote Env incorporation, and the role of specific membrane microdomains in this process. Here we review our current understanding of HIV-1 Env glycoprotein trafficking and incorporation into virions.

A. Overview of HIV-1 replication

In vivo, human immunodeficiency virus type 1 (HIV-1) infects predominantly $CD4^+$ T cells and cells of the monocyte/macrophage lineage. This cell tropism, which is intimately linked to the pathogenesis induced by HIV-1, is determined by the viral envelope (Env) glycoproteins. The Env glycoprotein complex projects from the virion surface as highly glycosylated spikes, composed of a heterotrimer of the surface (SU) glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41. The entry process is initiated by the binding of gp120 to CD4, the primary attachment receptor for primate lentiviruses. CD4 binding triggers a conformational change in gp120 that enhances its affinity for a secondary receptor, or “coreceptor”. The major physiologically relevant coreceptors for HIV-1 are CCR5 and CXCR4. Upon binding of gp120 to the coreceptor, further conformational changes in both gp120 and gp41 trigger a membrane fusion reaction that delivers the viral core into the cytoplasm. HIV-1 can enter host cells through two different mechanisms, either direct, pH-independent fusion with the plasma membrane or clathrin-mediated endocytosis followed by low-pH fusion with the endosomal membrane.[1,2,3]

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The binding affinity between gp120 and CCR5 or CXCR4 to a large extent determines the cell tropism of a particular viral isolate. Macrophage-tropic HIV-1 isolates utilize CCR5 for entry and are thus designated as R5 viruses; these isolates can also infect primary $CD4^+$ T cells but not T-cell lines. Viruses that primarily use CXCR4 are denoted X4-tropic; these strains can infect T-cell lines and primary T cells. R5X4 or "dual-tropic" strains can enter cells via either CCR5 and CXCR4.[4] The CCR5 coreceptor is used during the primary or early, asymptomatic stage of HIV-1 infection and R5 viruses are largely responsible for person-to-person transmission.[5] During the late stage of infection, viral isolates may arise that use CXCR4 for entry. The emergence *in vivo* of X4 or dual-tropic viruses is often associated with a sharp decline in the number of $CD4^+$ T cells in infected individuals and the onset of AIDS-defining symptoms.

Following entry into the cytosol, the viral RNA genome is converted (reverse transcribed) into double-stranded DNA by the viral enzyme reverse transcriptase (RT). The newly synthesized viral DNA is then translocated across the nuclear pore as part of a high-molecular-weight structure known as the preintegration complex.[6,7,8,9,10] In the nucleus, the viral DNA is integrated into the host cell chromosomal DNA; this integration process is catalyzed by a second viral enzyme, integrase (IN). The integrated viral DNA directs the transcription of viral RNAs, which are transported into the cytoplasm where translation of the viral proteins takes place. The newly synthesized viral proteins, together with two single-stranded copies of full-length (unspliced) viral RNA, assemble into a new generation of viral particles. Concomitant with release of virus particles from the infected cell, a third viral enzyme, protease (PR), cleaves the Gag and GagPol polyprotein precursors, triggering the conversion of the immature particle to the mature virion. The virus replication cycle is now complete, and the mature virus particle can initiate a new cycle of infection. [11,12]

B. Introduction to HIV-1 assembly

The process of HIV-1 assembly is regulated by both viral and cellular factors. The Gag polyprotein precursor, $Pr55^{Gag}$, is the major viral structural protein responsible for assembly; its expression is sufficient for the assembly, budding, and release of immature particles. $Pr55^{Gag}$, usually simply referred to as Gag, is synthesized on cytosolic ribosomes and is composed of matrix (MA), capsid (CA), nucleocapsid (NC), and p6 domains, along with two spacer peptides (SP1 and SP2). Assembly of viral genomic RNA, the Env glycoprotein complex, and GagPol precursor protein ($Pr160^{GagPol}$) into virus particles occurs in most cells at the plasma membrane (PM).[12,13,14,15]

Each of the major domains in Gag (MA, CA, NC, and p6) serves distinct functions during the viral assembly process. Intracellular trafficking of $Pr55^{Gag}$ and binding of Gag to the PM is regulated by MA. Membrane association is mediated by a bipartite membrane-binding domain, comprised of a covalently attached myristic acid linked to the N-terminus of MA and a highly basic patch of residues that forms a positively charged surface at the "top", or membrane-proximal surface, of the folded MA domain.[16,17,18] One of the primary functions of the basic patch is to bind the PM phosphoinositide phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], which plays a major role in directing Gag to the PM.[19,20] The MA domain also directs the incorporation of the Env glycoprotein complex into virions, as will be described in more detail below. CA contributes to Gag-Gag interactions during assembly and ultimately forms the outer shell of the viral core after virion release and maturation. The NC domain mediates the packaging of viral genomic RNA and also promotes Gag multimerization. The p6 domain stimulates the release of viral particles from the PM by recruiting the endosomal sorting complexes required for transport (ESCRT) machinery, a cellular apparatus that normally functions in cellular budding and membrane scission events.[21,22,23,24,25]

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The question of where in the cell HIV-1 assembles has been the focus of debate in recent years. It is now clear that the major site of HIV-1 assembly is the PM.[26,27,28,29] However, under certain circumstances, late endosomes can function as the site of productive virus assembly.[30] In primary macrophages, it has long been appreciated that internal compartments that bear late endosomal markers (*e.g.*, the tetraspanins) are the primary sites of virus assembly. Several studies have demonstrated that these internal, virus-containing compartments are not true late endosomes but rather are specialized PM invaginations into which virus assembles and buds.[28,31,32] Virus particles contained in these internal, but PM-connected, compartments are rapidly released to the surface at sites of cell-to-cell contact known as virological synapses.[33]

C. Env biogenesis

Env synthesis and trafficking

HIV-1 Env glycoproteins are synthesized as a polyprotein precursor from a singly spliced, bicistronic *vpu*/*env* mRNA on the rough endoplasmic reticulum (RER) (Fig. 1).[34,35] The unprocessed Env glycoprotein precursor (gp160) contains an ER signal sequence at its N-terminus, which targets Env to the RER membrane. This signal peptide is cotranslationally removed by cellular signal peptidases within the ER. The transmembrane domain (TMD) of gp41 contains a hydrophobic stop-transfer signal (membrane anchor) that prevents gp160 from being fully released into the lumen of the ER.[36,37] The extracellular portions of Env thus extend into the lumen of the ER, while the gp41 cytoplasmic tail (CT) extends into the cytoplasm and later the interior of the virion. Concomitant with translation, gp160 is glycosylated with *N*-linked and some *O*-linked oligosaccharide side chains.[38,39,40] Monomers of gp160 oligomerize in the ER into predominantly trimers, although dimers and tetramers can also be observed.[41,42,43,44] This oligomerization process is thought to facilitate the trafficking of gp160 to the Golgi complex. As gp160 traffics through the secretory pathway, high-mannose oligosaccharide side chains acquire complex modifications in the trans-Golgi network (TGN). In part to prevent premature interaction of the oligomerized Env with CD4 in the secretory pathway, the HIV-1 accessory protein Vpu binds to CD4 and down-regulates its expression via ubiquitin-mediated proteasomal degradation.[45,46,47]

In the Golgi, gp160 is proteolytically cleaved by cellular furin or furin-like proteases[48] at a highly conserved K/R-X-K/R-R motif,[49,50] to yield the mature surface (SU) glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41. Proteolytic processing of gp160 is absolutely required for activation of the fusogenic activity of Env and is thus essential for viral infectivity. Following cleavage, gp120 and gp41 remain associated by noncovalent interactions. Three molecules each of gp120 and gp41 form a heterotrimeric HIV-1 glycoprotein spike. After its arrival at the PM, Env is rapidly recycled via endocytosis,[51,52] driven by interactions with the cellular clathrin adaptor complexes (see below). The internalization of Env and the shedding of gp120 from the cell surface due to the relatively weak and non-covalent nature of the gp120-gp41 interaction are factors that contribute to relatively low levels of Env incorporation into virus particles (~10 spikes/virion).[53] The maintenance of low cell-surface and virion-associated levels of gp120 and gp41 likely help HIV-1 evade the host immune response and decrease virus-induced cytopathicity.

HIV-1 spread from one cell to another occurs much more efficiently at points of cell-cell contact known as infectious or virological synapses (VS)[54,55,56,57,58] than spread as cell-free virus.[59] Env and Gag both accumulate at the VS, along with actin, CD4, coreceptors, adhesion molecules, and tetraspanins.[56,60,61,62,63] Env–receptor contacts have been reported to be required for VS formation induced by HIV-1 in T cells[60] and by murine leukemia virus (MLV) in fibroblasts.[64,65] In the MLV system, the Env glycoprotein appears to direct Gag assembly to the VS in a manner dependent on the CT of Env.[66] In macrophages, as in T

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cells, both Gag and Env accumulate at the VS but Gag localization to the site of cell-cell contact is fairly Env-independent.[33,67] How Env is directed to the synapse remains a question of central importance, given the vital role that HIV-1 transfer across the VS is likely to play *in vivo*.

Env Structure

gp120—Comparison of *env* gene sequences from diverse HIV-1 isolates identified a high degree of variability in discontinuous segments within the gp120 subunit. gp120 has five variable domains (V1-V5) interspersed with five relatively constant domains (C1-C5) (Fig. 2).[68,69] Also, a number of highly conserved Cys residues are located throughout gp120 and gp41. These Cys residues form intramolecular disulfide bonds that are crucial to the formation of Env tertiary structure (Fig. 3).[39] Typically, gp120 contains 18 Cys residues that are covalently bound to form nine disulfide bridges. V1 is separated from V2 by two disulfide bonds but the two loops are contained in a larger loop by yet another disulfide bond; V3 and V4 loops are also delimited by a disulfide bond. Several studies have reported changes in the number and distribution of Cys residues that could cause changes in Env structure and antigenicity.[70,71]

Approximately half of the molecular mass of gp120 is composed of *N*-linked glycans[38] with a small additional contribution from *O*-linked sugars.[40] There are 20-35 *N*-linked glycosylation sites in gp120 and 3-5 in gp41 that mask Env from host immune recognition,[72] contribute to Env folding,[73] and help virions bind to the host cell surface.[74]

Sequence variability in the V domains arose through recombination, point mutations, insertions and deletions. The V1V2 domain is the most variable in loop length and number of glycosylation sites.[75,76,77,78,79,80] The V1V2 loop can range in length from 50-90 amino acids (aa), while the length variation of V4 and V5 loops ranges from 19-44 aa and 14-36 aa, respectively. The V3 loop and C2, C3, and C4 domains show relatively little length variation. Furthermore, a correlation exists between an increase in length and number of glycosylation sites in V1V2 and disease progression, suggesting that modification of V1V2 length may promote escape from the host humoral immune response.[81]

The CD4 receptor binding site is formed from conserved residues in discontinuous segments that are folded into proximity in the Env tertiary structure. The conserved domains within gp120, in particular C1, C3 and C4, are the principle Env determinants that bind CD4.[82,83,84] The variable domains, which are exposed on the gp120 surface, do not have a direct role in CD4 binding, as a gp120 variant from which V1, V2, and V3 domains are deleted still binds to CD4 with high affinity.[85,86] A number of studies have shown that the V3 loop is important for membrane fusion,[87] coreceptor specificity,[88,89,90,91,92] and contains dominant epitopes recognized by neutralizing antibodies.[93,94,95,96] The switch from R5 to X4 tropism driven by V3 mutation has been linked to an increase in the net positive charge of V3, allowing an interaction with the negatively charged surface of CXCR4.[97,98,99] Mutations in the tip and base of V3 as well as changes in the so-called “bridging sheet” of gp120 (see below) can influence coreceptor binding.[100,101,102,103] The V3-CCR5 interaction is the target of the FDA-approved drug maraviroc, which binds directly to CCR5 to prevent gp120 association.[104] As one would expect for a CCR5-based drug, maraviroc is not active against viral isolates that enter via CXCR4. Interestingly, resistance to maraviroc arises not by switching coreceptor use to CXCR4 but primarily by acquiring mutations that allow gp120 to effectively utilize drug-bound CCR5.[105,106]

A number of gp120 structures have been solved by X-ray crystallography. These include the HIV-1 gp120 “core” (from which N- and C-termini and V1/V2 and V3 variable loops were deleted) in complex with fragments of CD4 and a neutralizing antibody;[107] the SIV

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"unliganded" gp120 (not bound to CD4);[108] HIV-1 gp120 unliganded and CD4-bound[109]; the gp120 core with V3 loop, bound to CD4 and neutralizing antibody;[110] the gp120 core locked in the CD4-bound conformation associated with a CD4 binding-site-specific neutralizing antibody;[111] the gp120 core in complex with poorly neutralizing CD4 binding-site-specific antibodies;[112] and the gp120 core bound to the gp120-interacting portion of gp41.[113] These studies have provided a wealth of information on the mechanism of gp120 interactions with CD4, coreceptor, gp41, neutralizing antibodies, and entry inhibitors. The overall fold of CD4-bound gp120 consists of an "inner" domain and an "outer" domain (so named for their putative orientation in the trimeric complex) linked by a four-stranded "bridging sheet" (Fig. 4A, B).[107] CD4 is observed to directly contact 26 residues in gp120;[107] these are widely dispersed in gp120, consistent with previous mutagenesis studies.[83,84,114] Comparison of CD4-bound and unliganded gp120 structures suggests that gp120 undergoes a remarkable degree of refolding following CD4 binding. This large CD4-induced conformational change results in the formation of the coreceptor binding surface from residues that were spatially well separated prior to CD4 binding.[108] This strategy allows the virus to delay the formation of the vulnerable coreceptor binding surface until the Env complex is in close contact with the target cell, at which time steric occlusion prevents effective access by neutralizing antibodies. Fitting the X-ray data into density maps obtained by electron tomography allows for models to be constructed for the gp120 trimer in unliganded and CD4-bound states (Fig. 4C, D).[115] The picture that emerges from these structural studies is that the HIV-1 Env glycoprotein complex is exquisitely evolved to keep key functional surfaces hidden from antibody recognition and sufficiently plastic and tolerant of sequence variation to escape a robust host immune response.

gp41—The gp41 TM glycoprotein mediates fusion of the viral envelope with host cell membrane. The gp41 subunit is comprised of ~345 aa, organized into three major domains: an extracellular domain (or ectodomain), a transmembrane domain (TMD), and a C-terminal cytoplasmic tail (CT) (Fig. 2). The extracellular domain contains the major fusion determinants: an N-terminal hydrophobic region known as the fusion peptide,[116,117,118] a polar region, two hydrophobic regions that form α-helical coiled-coil structures referred to as the heptad-repeat regions HR1 and HR2 (also known as N-helix and C-helix, respectively),[119,120,121,122,123] and a Trp-rich domain referred to as the membrane-proximal external region (MPER).[124,125] HR1 and HR2 are connected by a disulfide-bridge within a hydrophilic loop and their interaction drives the fusion process. The fusion peptide is normally buried in the gp120/gp41 quaternary complex. Binding of gp120 to CD4 and coreceptor changes the structure of gp41 leading to exposure of the fusion peptide and its penetration into target cell membrane, thus causing membrane destabilization[126] and formation of the fusion pore. Three HR1 motifs form a core bundle in parallel and fold over a hydrophobic groove antiparallel to three HR2 domains within each trimer, thus forming a stable six-helix bundle that brings the viral and cell membranes into close enough proximity for fusion to occur.[119,123] General structural features of the gp41-induced fusion mechanism are shared with those of other viral fusion proteins (*e.g.*, hemagglutinin of influenza)[127] and some cellular fusion proteins (*e.g.*, the SNAREs).[128] Formation of the gp41 six-helix bundle can be blocked by peptides derived from either HR1 or HR2.[129,130,131] One such HR2-derived peptide, known as enfuvirtide, T-20, or fuzeon, is approved for use in HIV-1-infected patients. Its high cost and lack of oral bioavailability, however, limit its clinical application.[132]

The MPER consists of the last 24 amino acids of the gp41 extracellular domain. This region is required for fusogenicity and virus infectivity;[124,125,133] however, the mechanism by which it functions in promoting membrane fusion is not entirely clear. The MPER is highly conserved and is recognized by several well-studied neutralizing antibodies (2F5, 4E10, and Z13).[134,135]

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The gp41 TMD consists of ~25 highly conserved amino acids that anchor Env in the lipid bilayer. The highly conserved nature of the gp41 TMD suggests that it plays specific roles in Env function, and, indeed, mutations in the "core" region of the TMD influence Env-mediated fusion.[136,137,138] The traditional model for gp41 topology proposes that the TMD is an α-helix that spans the membrane once and the CT is inside the virus particle (Fig. 5A). An alternative topology has been proposed in which gp41 spans the membrane three times, initially based on recognition of an epitope within the gp41 CT by neutralizing monoclonal Abs (Fig. 5B, "Kennedy" sequence). [139,140,141,142] Similarly, Lu *et al.* recently demonstrated that the LLP-2 region in gp41 CT may be transiently exposed on the cell surface during cell-cell fusion.[143] However, more recent studies suggest that the traditional "single-pass" model most accurately reflects the topology of gp41 in virions. For example, antibodies that bind the Kennedy sequence on Env-expressing cells do not bind Env on intact virions.[144] Furthermore, viral PR cleavage sites flanking the Kennedy sequence were acquired during selection for HIV-1 resistance to the cholesterol-binding compound amphotericin B methyl ester.[145,146,147] The ability of PR to cleave this sequence in virions strongly implies that this region of gp41 must be inside the virus particle.

The TM subunit of most lentiviral Env glycoproteins has a very long CT compared to those found in other retroviruses (Fig. 6).[34] For example, the gp41 CTs from HIV-1, HIV-2, and SIVs are approximately 150 aa in length; the equine infectious anemia virus (EIAV) CT is even longer (~200 aa).[148,149] In contrast, the TM CTs of other prototypic retroviruses, such as Rous sarcoma virus (RSV, an alpharetrovirus), Mason-Pfizer monkey virus (M-PMV, a betaretrovirus), and murine leukemia virus (MLV, a gammaretrovirus), are only ~20-40 aa long (Fig. 6). The presence of a very long CT in the vast majority of lentiviral TM Env glycoproteins suggests that they evolved to serve specific functions in lentiviral replication, a hypothesis supported by the observation that truncation of the lentiviral CT leads to suppression of virus replication in animal models.[150] The CT of HIV-1 gp41 influences multiple properties of the gp120/gp41 glycoprotein complex, such as Env incorporation into virus particles, virus infectivity, cell-surface Env expression, gp120 shedding, and Env-induced fusion[151,152,153,154,155,156,157,158,159,160,161]; discussed in more detail below). The CTs of HIV-1 and SIV gp41 also influence the conformation of gp120 and the ectodomain of gp41 such that CT mutations affect antibody recognition and neutralization.[162,163,164,165] It is interesting to note that some HIV-1 isolates that have acquired CD4 independence bear truncations in their gp41 CTs,[163,166] again consistent with a role for this domain of Env in regulating gp120 conformation.

The activities of the gp41 CT are modulated by several functional and structural domains (Fig. 2, 5). A membrane-proximal tyrosine-based sorting signal with the consensus sequence YxxL interacts with the clathrin adaptor protein complex 2 (AP-2) to mediate clathrin-dependent endocytosis of Env from the PM.[167,168,169,170] This motif has also been implicated in the targeted release of virions from the basolateral surface of polarized epithelial cells, [171] cell-cell transmission in T cells,[172] and particle infectivity.[173,174] Mutation of the analogous signal in SIV resulted in attenuation of virus replication *in vivo*.[175] The intracellular distribution of Env is also regulated by a dileucine motif in the gp41 CT; this signal interacts with the clathrin adaptor protein complex 1 (AP-1).[155,170,176] Endocytosis of Env via this motif is independent of the function exerted by the YxxL motif. Mutation of both the C-terminal dileucine motif and the YxxL motif is sufficient to completely inhibit cell surface downregulation of Env, suggesting that both motifs account for most Env internalization.[176]

Three conserved amphipathic α-helical segments that are referred to as lentiviral lytic peptides (LLP-1, LLP-2, and LLP-3) are present in the central and C-terminal regions of the gp41 CT (Fig. 2, 5).[177,178,179,180] LLP-1 and LLP-2 segments are highly positively charged

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due to the presence of Arg residues on one face of the α-helix. LLP-3 is located between the other two segments. The structure of the LLPs is highly conserved among lentiviruses.[177] LLP domains have been implicated in Env fusogenicity,[160] protein stability,[181] multimerization,[182] cell surface expression,[183] and incorporation.[159,184,185] Also, LLP fragments have been shown to bind and perturb membranes,[186,187,188,189] causing cytolysis, hence the name "lytic peptides".[178] Mutation of the Arg residues in LLP-1 to Glu, which allows the preservation of the secondary structure and hydrophilicity of this segment, results in loss of cell lysis activity, implying that these residues are important for LLP function.[190] Although the results are somewhat controversial, the CT contains two Cys residues that are palmitylated, a post-translational modification that has been proposed to target Env to lipid rafts.[191] The LLP-1 segment has also been proposed to contain the determinants for lipid raft association, as truncation of LLP-1 led to a decrease in localization of Env to lipid rafts[192] (see below for further discussion of this topic). It is clear from the discussion above that the biological functions of the gp41 CT are diverse and somewhat enigmatic; a more complete understanding of this region of Env will require careful analysis of its biological properties in relevant primary cell types, evaluation of its role in cell-cell transfer, and the identification of cellular interaction partners.

D. General models for HIV-1 Env glycoprotein incorporation

Several general models can be invoked to explain the incorporation of HIV-1 Env glycoproteins into virus particles: 1) The "passive" or "random" incorporation model (Fig. 7A) postulates that the Env glycoprotein complex is incorporated into virions simply as a result of its expression on the host cell PM. This model requires no mechanism for concentrating Env at sites of virus assembly. 2) The "direct Gag-Env interaction" model (Fig. 7B) proposes that direct binding between the MA domain of Gag and the CT of gp41 recruits Env glycoprotein spikes into virus particles. 3) The "Gag-Env cotargeting" model (Fig. 7C) postulates the existence of a cellular structure (*e.g.*, a PM microdomain) that acts as an assembly platform to which both Gag and Env are recruited, thereby enhancing the efficiency of Env incorporation. 4) The "indirect Gag-Env interaction" model (Fig. 7D) invokes the existence of a host cell protein that serves as a bridge between Gag and Env to promote Env incorporation.

Passive incorporation model

The passive model draws support from the observation that host cell membrane proteins are abundantly incorporated into virus particles. In contrast to some enveloped viruses that tightly restrict the packaging of host proteins (*e.g.*, the alphaviruses),[193,194] retroviruses are fairly promiscuous in their virion incorporation of host membrane proteins.[195,196,197] Furthermore, non-HIV viral glycoproteins can be readily incorporated into HIV-1 virions to generate pseudotyped particles that display high levels of particle infectivity. For example, amphotropic murine leukemia virus (ampho-MLV) Env and vesicular stomatitis virus G glycoprotein (VSV-G) are frequently used to pseudotype HIV-1 particles.[198,199] Finally, a number of studies have shown that removal of most or virtually all of the gp41 CT has only a modest effect on Env glycoprotein incorporation into HIV-1 particles produced in some commonly used laboratory cell lines (*e.g.*, HeLa, 293T, CV-1, and COS).[152,156,157,158,200] Based on these findings, it would seem plausible that passive incorporation could be sufficient to account for the ~10 copies[53] of gp120/gp41 heterotrimeric spikes present on HIV-1 virions.

Direct Gag-Env interaction model

Despite the apparent lack of a requirement for the long gp41 CT in Env incorporation in some laboratory cell lines, a number of studies demonstrated that the MA domain of Gag is

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required for Env incorporation.[156,157,201,202] Deletion of the gp41 CT reversed the Env incorporation defect imposed by mutations in MA,[156,157,203] or MA deletion,[204] suggesting that incorporation of full-length Env requires an interaction with MA, whereas incorporation of truncated Env does not require Env-MA binding. Although deletion of the entire gp41 CT has little effect on Env incorporation in HeLa and 293T (COS) cells, somewhat paradoxically, smaller deletions or truncations within the tail lead to lower levels of Env in virus particles.[153,154,159,185,205,206] Although in some cases the effects of these gp41 CT mutations can be attributed to reduced cell-surface Env expression, in other cases the CT mutations appear to specifically impair Env incorporation. Interestingly, the effects of a gp41 CT deletion that reduced Env incorporation could be reversed by a point mutation in MA that was acquired during propagation of the gp41 mutant virus in culture.[159]

X-ray crystallography studies revealed that HIV-1 and SIV MA form a trimeric lattice containing holes that could potentially accommodate a trimer of gp41 CTs.[18,207] This led to the model that the gp41 CTs might project into these holes, thereby permitting direct contacts to take place between residues in MA and gp41. Indeed, several key residues shown to be required for Env incorporation (MA aa 12, 30, and 34)[156,157,159,208] appear to face the inside of the largest hole in the trimeric lattice. While this is still a viable model, there is currently little evidence supporting the formation of MA trimers in virions. Also, uncertainty remains about the orientation of the gp41 CT with respect to Gag; i.e., do the CTs “swim” in the plane of the membrane or do they project toward the center of the virion parallel to the radial orientation of Gag? It is also possible that MA-gp41 interactions, and perhaps the orientation of gp41 CTs relative to Gag, change during the transition from immature to mature particles that accompanies PR-mediated Gag processing.

Evidence of cross-talk between Gag and gp41 during assembly also derives from studies of Gag and Env localization in polarized epithelial cells. HIV-1 Env was shown to localize to the basolateral surface of Madin-Darby canine kidney (MDCK) polarized epithelial cells.[209] In the absence of Env expression, Gag budded in a non-polarized manner in this cell type. However, when Env and Gag were coexpressed, budding was restricted to the basolateral surface.[210] Mutations in MA that prevented Env incorporation, as well as truncations in the gp41 CT, reversed the ability of Env to direct basolateral budding of Gag.[211] Polarized budding reportedly requires the Tyr-based (YxxL) sorting signal located in the gp41 CT close to the transmembrane domain.[171] Together, these findings are consistent with the concept that Env and MA associate in some manner during virus assembly.

Additional findings provide support for the concept that MA and Env (specifically the gp41 CT) interact in virions. Mature virions, which contain fully processed Gag proteins, can fuse with the target cell membrane. This virion-cell membrane fusion activity can be detected in an assay in which virions are engineered to package the enzyme β-lactamase; upon fusion of the virion with target cells, β-lactamase is delivered into the target cell cytoplasm. This delivery of β-lactamase can be quantitatively detected by preloading the target cells with a fluorogenic β-lactamase substrate. In contrast to the robust virion-cell fusion activity observed with mature virions, immature virions, in which Gag remains unprocessed, are fusion defective.[212] A similar defect in Env-mediated membrane fusion activity in immature virions can be measured in a “fusion-from-without” assay in which high concentrations of virions induce cell-cell fusion.[213] In both of these studies, the fusion defect observed with immature particles could be reversed by full gp41 CT truncation,[212,213] or, in a subsequent study, by removal of the C-terminal 28 amino acids of gp41.[214] These results suggest that an interaction between the gp41 CT and uncleaved Gag locks Env in a non-fusogenic state; cleavage of Gag by the viral PR during virion maturation then activates the fusogenic activity of the Env glycoprotein complex. The suppression of Env-mediated fusion by uncleaved Gag could provide a mechanism for keeping fusion activity in check until virion

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maturation occurs, when the particle is ready to infect a new target cell. Intriguingly, virion maturation is reportedly accompanied by a significant reduction in particle "stiffness", measured by nanoindentation atomic force microscopy methods.[215] The degree of "stiffness" of the virus particle in this study was regulated by the gp41 CT, raising the possibility that this physical feature of the virion is influenced by Gag-gp41 interactions.[215]

Whereas HIV-1 modulates Env function via PR-mediated Gag processing (*i.e.*, virion maturation), several other retroviruses have evolved an alternative strategy for regulating the fusogenicity of the Env glycoprotein complex until PR-mediated virion maturation takes place. In MLV, EIAV, and M-PMV particles, the viral PR cleaves the CT of the transmembrane Env protein. This PR-mediated cleavage of the Env CT is required for activating the fusogenicity of the Env glycoprotein complex. PR-mediated cleavage of the Env protein can occur *in trans;* MLV Env used to pseudotype HIV-1 virions undergoes HIV-1 PR-mediated processing of its Env CT. Interestingly, this PR-mediated Env cleavage can be blocked by specific mutations in MA.[216] The simplest explanation for these results is that the MA mutations change the conformation of the MA shell underlying the lipid bilayer, thereby restricting the ability of PR to access and cleave the MLV Env CT. Although WT HIV-1 Env has not been observed to undergo PR-mediated gp41 CT processing, HIV-1 variants that have escaped the fusion-inhibitory activity of the cholesterol-binding compound AME acquire gp41 CT mutations that create novel cleavage sites for PR.[146,147] PR-mediated cleavage of the gp41 CT allows virion-cell membrane fusion to proceed even in the presence of AME.

Evidence for modulation of Env function by MA also derives from a study demonstrating that a mutation in MA influences the association between gp120 and gp41, leading to increased shedding of gp120 from the virus particle.[217] Again, the ability of MA to influence gp120 shedding requires the presence of an intact gp41 CT.[217]

A common theme in many of the studies discussed above is the apparent cross-talk between the MA domain of Gag and the gp41 CT. Despite the abundant circumstantial evidence in support of a MA-gp41 CT interaction, only a limited amount of supportive biochemical data have been obtained. Cosson reported a direct *in vitro* binding between the gp41 CT fused to glutathione-S-transferase (GST) and MA bound to β-Gal.[184] Although the binding data were reinforced by the observation that mutations in both Env and MA that disrupt Env incorporation reduced the interaction, these results have not been independently confirmed. Similarly, Hourioux *et al.*[218] used peptides representing the central region of HIV-1 gp41 CT (flanking LLP-2) to inhibit the immunocapture of membrane-free $Pr55^{Gag}$ particles by immobilized anti-$p24^{CA}$ antibody *in vitro*, again implying a direct Env-Gag interaction. Wyma and colleagues[219] demonstrated that gp41 is retained in immature virus particles that were treated briefly with detergent to solubilize the viral lipid bilayer. The retention of gp41 and some gp120 in these detergent-stripped immature particles required the gp41 CT, consistent with the existence of an interaction (direct or indirect) between Gag and gp41. While successful co-immunoprecipitation of Gag and Env in HIV-1-expressing cells has not been reproducibly achieved, Vincent *et al.* reported such data for SIV.[220]

Gag-Env co-targeting model

Although, as discussed above, a considerable number of reports support the concept that the gp41 CT and MA interact during assembly to promote the recruitment of Env complexes into nascent virions, most of these findings do not distinguish between a direct gp41 CT-MA interaction and an indirect interaction mediated by a host cell protein or membrane structure. A compelling piece of evidence to support the role of host cell factor(s) in Env incorporation is the observation that the requirement for the gp41 CT in Env incorporation and virus replication is strongly cell-type-dependent. As mentioned above, in several popular

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laboratory cells lines (*e.g.*, HeLa, COS, 293T), and in a small number of T-cell lines (notably MT-4 and M8166), the gp41 CT can be entirely deleted with little effect on Env levels in virions.[152,158,221] These cell lines are referred to as being "permissive" for gp41 truncation. In contrast, the majority of T-cell lines and the more physiologically relevant primary cell types (T cells and monocyte-derived macrophages) are "non-permissive" for gp41 truncation; in these cells, removal of the gp41 CT induces a ~10-fold defect in Env incorporation and a complete block in virus replication.[158] The basis for this cell-type-dependent requirement for the gp41 CT remains to be defined. A number of cellular factors (discussed in more detail below) have been reported that bind the gp41 CT; differences in the expression or localization of one or multiple host factors could account for the cell type dependence. SIVmac also displays interesting cell-type-dependent requirements for the gp41 CT in virus replication. When grown in human cells, SIVmac typically acquires truncations in the gp41 CT that facilitate efficient virus replication.[222,223] When SIVmac variants with short CTs are passaged in monkey cells in culture or *in vivo*, strains encoding full-length gp41 predominate.[222,223,224] As is the case for the HIV-1 gp41 truncations, the basis for this cell-type dependence remains to be defined; Vzorov *et al.* have suggested that gp41 truncation affects multiple stages of the virus replication cycle, including early, post-entry events.[225]

The host cell mediator of indirect Env incorporation could be a protein factor (discussed in more detail below) or a membrane microdomain or other cellular structure to which both Gag and Env are targeted. Membrane microdomains known as "lipid rafts",[226] which are enriched in cholesterol and highly saturated lipids (*e.g.*, sphingolipids), have been studied extensively over the past two decades.[227] Although somewhat controversial,[228] there is now widespread agreement that cholesterol- and saturated lipid-enriched raft microdomains do exist and serve important functions in a variety of cellular processes.[227] Lipid rafts have also been reported to promote the replication of a number of enveloped viruses, including retroviruses, by serving as organizing platforms for both virus entry and particle assembly (for reviews, see Refs.[229,230,231]). The highly saturated nature of lipid rafts causes them to be less soluble in cold non-ionic detergent (typically Triton X-100) than non-raft membrane.[232] Although detergent-resistant membranes (DRM) isolated from lysed cells are unlikely to represent native membrane structures found in living cells,[228] the presence of proteins in DRM is often used as evidence of their raft localization.[233,234] Studies performed in the early 2000's showed that both HIV-1 Gag and, to a lesser extent, HIV-1 Env, are DRM-associated.[191,235,236,237,238] Evidence that HIV-1 Gag and Env are raft associated also derives from microscopy analyses demonstrating copatching of Gag, Env, and cellular raft components.[229,237,239] Furthermore, treating virus-producing cells with cholesterol-depleting, binding, or sequestering agents impairs HIV-1 particle production.[145,236,237,240,241] In the case of cholesterol depletion, the defect in virus assembly and release is associated with reduced Gag–membrane binding and higher-order Gag multimerization.[240] Lipid rafts often function as platforms for promoting protein–protein interactions; protein multimerization in turn stabilizes lipid rafts and induces their aggregation.[227] Newly synthesized HIV-1 Gag appears to bind membrane rapidly, then more slowly acquires raft-associating properties, concomitant with Gag assembly and raft aggregation.[236,242] Also consistent with the hypothesis that HIV-1 assembly takes place in cholesterol-enriched membrane microdomains is the observation that the viral envelope displays a rather striking raft-like composition (elevated levels of cholesterol and saturated lipids) relative to the host cell PM.[243,244]

The mechanism by which the HIV-1 Env glycoproteins associate with rafts and the domains required for this association have been somewhat controversial topics. An early report suggested that palmitylation of the gp41 CT is critical for both DRM association and Env incorporation. These findings were surprising given that the study was performed in 293T

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cells, and that as mentioned above, deletion of the entire gp41 CT has little effect on Env incorporation in this cell line.[158] Subsequent studies found that mutating the gp41 CT Cys residues that serve as palmitate attachment sites either had no effect on Env–DRM association, Env incorporation, or virus replication[245] or reduced DRM association with only a small effect on virus infectivity.[246] While these two studies differ somewhat in their findings, probably due to the use of different viral isolates and producer cell types, both concluded that palmitylation of the gp41 CT is unlikely to play a major role in virus replication efficiency, at least in culture. So what is the mechanism by which Env is recruited to lipid rafts? Bhattacharya and colleagues[247] reported that Gag directs the recruitment of Env glycoproteins to lipid rafts; Env co-expressed with Gag was found in DRM, whereas Env expressed in the absence of Gag was not DRM-associated. Likewise, mutations in either Env or MA that disrupt Env incorporation abrogated the putative raft association of Env. However, the role of Gag in targeting Env to rafts does not exclude a role for Env in polarized targeting of Gag. Many reports suggest that the membrane-proximal Tyr-based sorting signal in gp41 CT may polarize HIV release in some cell types.[171,172,211]

Two recent studies that examined the mechanism by which viral pseudotypes are generated have bearing on the Gag-Env co-targeting hypothesis. Leung *et al.*[248] investigated whether HIV-1 Env glycoproteins and the Ebola virus glycoprotein (GP), when expressed in the same cell, are copackaged into HIV-1 virions or segregated into two distinct populations of virus particles. HIV-1 virions produced from cells expressing both HIV-1 and Ebola GP were treated with neutralizing antibodies against each glycoprotein, immunodepletion analyses were performed, and colocalization between HIV-1 and Ebola GP was analyzed. The surprising and interesting result was that virions appeared to contain either HIV-1 Env or Ebola GP, but not both. This result suggests that HIV-1 Env and GP segregate into distinct microdomains on the cell surface, each of which associates with individual virions. The microdomains to which HIV-1 Env and GP segregate could be lipid rafts or some other type of microdomain or subcellular structure. Jorgenson and colleagues[249] examined the coclustering of three different retroviral Env glycoproteins – those of Rous sarcoma virus (RSV), MLV or HIV-1 – with RSV or HIV-1 Gag proteins. Scanning electron microscopy and immuno-electron microscopy showed that none of the viral glycoproteins demonstrated appreciable clustering in the absence of Gag expression. RSV Env clustered at budding sites in the presence of RSV but not HIV-1 Gag; the coclustering of RSV Env and Gag required the CT of RSV Env. MLV and HIV-1 Env both clustered with HIV-1 Gag at virus budding sites. These data support a role for Gag in recruiting Env to PM microdomains (lipid rafts or potentially other distinct structures) and highlight interesting differences in the segregation behavior of different retroviral Env glycoproteins. Direct interactions between Gag and Env from distantly related retroviruses seem unlikely, arguing for co-recruitment of Gag and Env to specific PM structures or microdomains. These data are also inconsistent with the passive incorporation model, according to which no mechanism for co-clustering of Gag and Env is required.

Indirect Gag-Env interaction model: gp41 CT-binding host factors

The Gag-Env co-targeting model discussed above posits that Gag and Env are recruited, by unknown mechanisms, to a common site on the PM (for example, a lipid raft). It also remains possible that, at least under some circumstances, a cellular protein could act as a bridge between MA and the gp41 CT to recruit Env complexes into virions. A number of host proteins have been reported to interact with MA and/or the gp41 CT.

The best understood binding partners of the gp41 CT are the clathrin adaptor protein complexes AP-1 and AP-2. These complexes direct the sorting and trafficking of proteins in the secretory and endocytic pathway. As mentioned earlier, AP-2 drives the clathrin-

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mediated endocytosis of Env from the PM by binding to the YXXL motif in the gp41 CT.[167,168,169,170] AP-1 mediates the trafficking of vesicles between the TGN and endosomes and regulates the subcellular localization of Env via binding to a dileucine motif in the gp41 CT (Fig. 2).[155,170,176] AP-1 and AP-2 have also been shown to bind HIV-1 Gag in the MA domain and at the MA-CA junction, respectively.[250,251]

Tail-interacting protein of 47 kDa **(**TIP47) was reported to play a direct role in Env incorporation. TIP47, also known as mannose-6-phosphate receptor (MPR) binding protein 1, was first described as having a role in the retrograde traffic of MPRs from late endosomes to the TGN[252] via binding to Rab9.[253] However, subsequent reports showed that TIP47 does not colocalize with MPR or Rab9, does not bind Rab9 in vitro, or affect the trafficking or function of MPRs. Instead, TIP47 appears to be involved in the biogenesis of lipid droplets,[254,255,256] although early reports were not entirely consistent.[257] Indeed, TIP47 is a member of the PAT [for perilipin/adipose differentiation-related protein (APRP)/TIP47] family of lipid droplet-associated proteins.[258] Berlioz-Torrent and colleagues identified an interaction between TIP47 and the gp41 CT by yeast two-hybrid and GST pull-down assays. [259] As further evidence of a TIP47-gp41 CT interaction, mutation of a YW diaromatic motif in LLP-3 of the gp41 CT resulted in decreased binding of TIP47 by GST pull-down and yeast two-hybrid assays and decreased targeting of Env to the TGN. YW motif mutation disrupted virus replication in the Jurkat T-cell line and induced a defect in Env incorporation. Deletion of the YW motif had previously been shown to delay virus replication and impair Env incorporation, defects that were corrected upon acquisition by the virus of a compensatory mutation in MA.[159] TIP47 was later proposed to act as a linker between the gp41 CT and MA, thus allowing efficient Env incorporation in both Jurkat and HeLa cells.[260] In this study, yeast two-hybrid and coimmunoprecipitation (coIP) assays demonstrated that TIP47 interacts with a WE motif near the N-terminus of MA. Lopez-Verges *et al.* also showed that coIP of gp41 by Gag was reduced when TIP47 was depleted. Subsequently, it was shown that HIV-1 with mutations in the Env YW or MA WE motifs displayed defects in replication and infectivity in monocyte-derived macrophages, providing indirect evidence that TIP47 is involved in the production of infectious HIV-1 particles in this primary cell type.[261] However, a role for TIP47 in HIV-1 biology has not been independently confirmed, and our own studies have failed to support a role for TIP47 in Env incorporation, particle production, or virus replication (Checkley and Freed in preparation). The role of TIP47 in HIV-1 replication thus remains uncertain.

Human discs large protein (Dlg1/hDlg1/Dlgh/synapse-associated protein 97) is a scaffolding protein implicated in the organization of multiprotein complexes at the immunological synapse.[262] Blot and colleagues[263] demonstrated that Dlg1 interacts with the CT of HTLV-1 Env and colocalizes with HTLV-1 Env and Gag in infected cells. In follow-up work, Perugi *et al.* investigated whether this protein functions in HIV-1 replication. They observed that Dlg1 binds HIV-1 Gag; Dlg1 depletion was found to upregulate HIV-1 Env expression and increase Env levels in virions. These effects correlated with an accumulation of both Gag and Env in putative late endosomal ($CD63^+$) intracellular compartments and PM-associated tetraspanin-enriched microdomains.[264] The mechanism responsible for the altered Gag and Env localization and the increased Env expression and incorporation induced by Dlg1 depletion remains to be elucidated.

Calmodulin, p155-RhoGEF, α-catenin, prenylated Rab acceptor (PRA1/PRAF1), luman, and the prohibitin 1/prohibitin 2 heterodimer have also been observed to interact with HIV-1 gp41, however a clear role for these proteins in Env function has not been established. HIV-1 gp41 and peptide sequences derived from LLP-1 and LLP-2 in the gp41 CT, which structurally resemble calmodulin-binding proteins, [179,265] have been shown to interact *in vitro* with calmodulin.[190,266,267,268] Calmodulin senses and binds calcium to function as a

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signal transducer. Increased Env levels are associated with upregulated calmodulin expression[269,270] and sensitivity to Fas-mediated apoptosis in certain cell types.[269,271,272] Based on these findings, the interaction of the gp41 CT with calmodulin has been proposed to sequester calmodulin and consequently lead to T cell anergy and apoptosis, which are linked to AIDS progression.[267,272,273] Recent studies have provided evidence that MA also binds calmodulin in a calcium-dependent manner *in vitro*.[274,275] Nuclear magnetic resonance (NMR) data revealed that upon binding to calmodulin MA undergoes a marked conformational change,[274,275] which triggers exposure of the myristic acid moiety covalently attached to the N-terminus of MA.[275] Myristate exposure is crucial for binding of Gag to membrane.[276]

Kim *et al*.[277] identified an interaction between gp41 from HIV-1 or SIV with the C-terminal domain of α-catenin in a yeast two-hybrid screen. α-catenin has been implicated in the regulation of actin filament assembly and dynamics[278] and proposed to play a role in cell-cell contact formation.[279] An interaction between the gp41 CT and α-catenin was later confirmed by *in vitro* binding assays and the binding domain was mapped to the LLP-3 domain of the gp41 CT.[280] HIV-1 Env was shown to colocalize with α-catenin at the PM of HeLa cells microinjected with Env and α-catenin expression vectors.[280]

LLP-3 has been reported to interact with the C-terminal regulatory domain of the guanine nucleotide exchange factor p115-RhoGEF.[281] This interaction may be physiologically important, as mutation of residues that abolish binding to p115-RhoGEF impairs HIV-1 replication. p115-RhoGEF activates RhoA GTPase, which stimulates actin stress fiber formation [282,283] and activates serum response factor (SRF), which in turn induces cell proliferation.[284,285,286] These p115 activities were impaired when the gp41 CT was expressed in murine fibroblasts.[281] A RhoA-derived peptide has been shown to inhibit CXCR4-dependent HIV-1 entry in culture;[287] however, this anti-HIV activity is linked to the polyanionic properties of the RhoA peptide, suggesting that the peptide may disrupt the infection process simply by interfering with gp120/CXCR4 binding, and not as a result of a requirement for RhoA binding during entry.

PRA1 is a Rab protein regulator that binds prenylated Rab GTPases to mediate the trafficking of Rabs in vesicles.[288] PRA1 localizes primarily to the Golgi[289,290] and interacts with Rab3A and vesicle-associated membrane protein 2 (VAMP2) to regulate vesicle docking and fusion in the Golgi complex and the PM.[291] PRA1 was first identified as a cellular binding partner of the SIV gp41 CT in a yeast two-hybrid screen.[292] In this same study, PRA1 also interacted with the Env CT from various lentiviruses, including HIV-1, EIAV, and FIV, in a mammalian two-hybrid system. However, the significance of this interaction is not clear. Despite the observation that PRA1 colocalizes with SIV gp41 in 293T cells, modulation of PRA1 protein levels by RNAi or overexpression does not affect SIV or HIV-1 release, infectivity, or Env incorporation.[293] Nonetheless, studies have also shown an interaction of PRA1 with proteins from other viruses, including a lipid raft-associated rotavirus spike protein (VP4)[294,295] and the LMP1 oncoprotein of Epstein-Barr virus[296] (reviewed in Ref.[297]).

Luman is an ER-membrane-bound transcription factor of the CREB3 family that is involved in ER stress responses, such as the unfolded protein response (UPR) and ER-associated degradation (ERAD). During ERAD, ER stress caused by an accumulation of misfolded proteins leads to cleavage of the cytoplasmic domain of luman, which translocates to the nucleus and activates factors that enhance proteasomal degradation.[298,299] In 2006, Blot *et al*.[300] identified an interaction between luman and the HIV-1 gp41 CT. Expression of Env bearing the gp41 CT led to a decrease in luman levels in cells. Luman was also observed to interact with Tat, and expression of a transcriptionally active form of luman led to a

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decrease in Tat-mediated HIV-1 gene expression.[300] Env expression could thus potentially counteract luman's ability to suppress HIV-1 gene expression. Deciphering the biological implications of these observations awaits further analysis.

Using tandem affinity purification methods, the endogenous prohibitin 1 and 2 (Phb1 and Phb2) heterodimer was shown to bind a tagged HIV-1 gp41 CT.[301] Phb1 and Phb2 are members of the prohibitin family of proteins, which are ubiquitously expressed and localize to diverse cellular compartments including the mitochondria, nucleus, lipid droplets, and the PM.[302,303] They are involved in mitochondrial function and morphogenesis, cell proliferation, and transcriptional regulation.[302,303] Prohibitin proteins contain the prohibitin homology domain, which is found in proteins known to associate with lipid rafts.[304] The interaction between gp41 and Phb1 and Phb2 was validated by several *in vitro* binding assays and mapped to LLP-3. HIV-1 mutants with reduced Phb1/Phb2-binding were replication defective in a cell line non-permissive for gp41 truncation mutants (H9) but were replication competent in the highly permissive MT-4 T-cell line.[301] However, the defective replication phenotype conferred by mutation of the putative Phb1/Phb2 binding site in gp41 was not associated with impaired Env incorporation.[301]

Conclusions

A key step in the production of infectious HIV-1 particles is the incorporation of the Env glycoprotein complex. Much is known about events leading to Env incorporation; however, the actual mechanism of incorporation still remains unclear. Four general models can be postulated (Fig. 7). The **passive incorporation** model, in which Env and Gag arrive at virus assembly sites independently and incorporation occurs simply as a result of gp120/gp41 expression on the cell surface. The **direct Gag-Env interaction** model, according to which binding of the gp41 CT to the MA domain of Gag mediates Env incorporation. The **Gag-Env co-targeting** model, in which Gag and Env are targeted to lipid rafts or other PM microdomains or structures. The **indirect Gag-Env interaction** model, which posits that Gag and Env associate with host proteins that serve as linkers or adapters to facilitate Env incorporation. Since these models are not mutually exclusive, it is possible that each contributes to Env incorporation to varying degrees in different cellular contexts.

Clearly distinguishing between these models will require progress in answering a number of key questions. For example, what is the structure and topology of the gp41 CT? While we now know quite a lot about the structures of gp120 and the ectodomain of gp41 at the atomic level, the structure of the gp41 CT, which plays a key role in Env incorporation, is still a matter of speculation. Although there is evidence for two, or several, distinct topologies of the gp41 CT in Env-expressing cells, the traditional model of a single membrane-spanning domain most likely reflects the conformation of the active Env complex in virions. But how are the LLP domains oriented with respect to the lipid bilayer? Do they associate with each other to form coiled-coil domains, do they “swim” in the plane of the membrane or are they oriented away from the membrane, do they bind directly to Gag and/or host factors, and does Gag processing and virion maturation alter their orientation? By analogy with gp120, which undergoes a number of major structural rearrangements during receptor/coreceptor binding and membrane fusion, one can imagine that the gp41 CT is also capable of adopting a variety of different conformations. Important advances have been made recently in our ability to solve the structures of membrane proteins by using techniques such as reverse micelle encapsulation[305] and electron crystallography. [306,307] Application of these tools to solving the structure of the CT of membrane-associated Env appears warranted.

A related question is what domains of the gp41 CT are required for Env incorporation? To date, essential domains in the gp41 CT have been identified by amino acid substitutions or

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deletions followed by biochemical analyses of Env incorporation efficiency. However, robust assays for detecting and quantifying Gag-Env interactions are still lacking, and many of these mutations affect Env trafficking and/or protein stability, suggesting that they may alter global folding. Developing a genetic screen that identifies essential domains in the gp41 CT may provide further insight into regions required for interaction with viral or host proteins.

It is clear that the role of the gp41 CT in Env incorporation is cell-type-dependent. What is the basis for this cell-type specificity? If it is based on the pattern of cellular factors expressed, then which of these promote Env incorporation? Alternatively, the cell type-dependent differences elicited by gp41 CT deletion may reflect variations in PM lipid composition and microdomain formation in different cell types. Both Gag and Env display properties of being associated with lipid rafts at the PM. What are the nature, composition, and properties of these Gag- and Env-recruiting microdomains, and how are Env glycoproteins from different viruses segregated into different microdomains?

Also of great interest is the question of how Gag-Env interactions affect their co-recruitment to the VS and subsequent early events in the next round of viral replication. Is there a role for gp41 at a post-entry step, after fusion is completed, perhaps as a trigger for uncoating? The many potential contributions of Gag to Env function (*e.g.*, fusogenicity, membrane localization in polarized cells, recruitment to the VS, gp120 shedding, and virus particle stiffness) need to be defined more completely.

Answers to these questions will not only enhance our understanding of Env incorporation but may open the way to the development of novel antiretrovirals that target Env incorporation. Given that diverse viruses likely utilize conserved mechanisms for directing Env incorporation, understanding this key step for HIV-1 would likely further our understanding of Env incorporation in other viral systems as well.

Acknowledgments

We thank P.D. Kwong, S. Subramaniam, A. Ono, J.D. Steckbeck, R. Montelaro, C.K. Leonard, and T.J. Gregory for permission to reproduce or adapt published figures. We are grateful to M. Johnson and members of Freed laboratory for helpful discussions. Research in our laboratory is supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and by the Intramural AIDS Targeted Antiviral Program.

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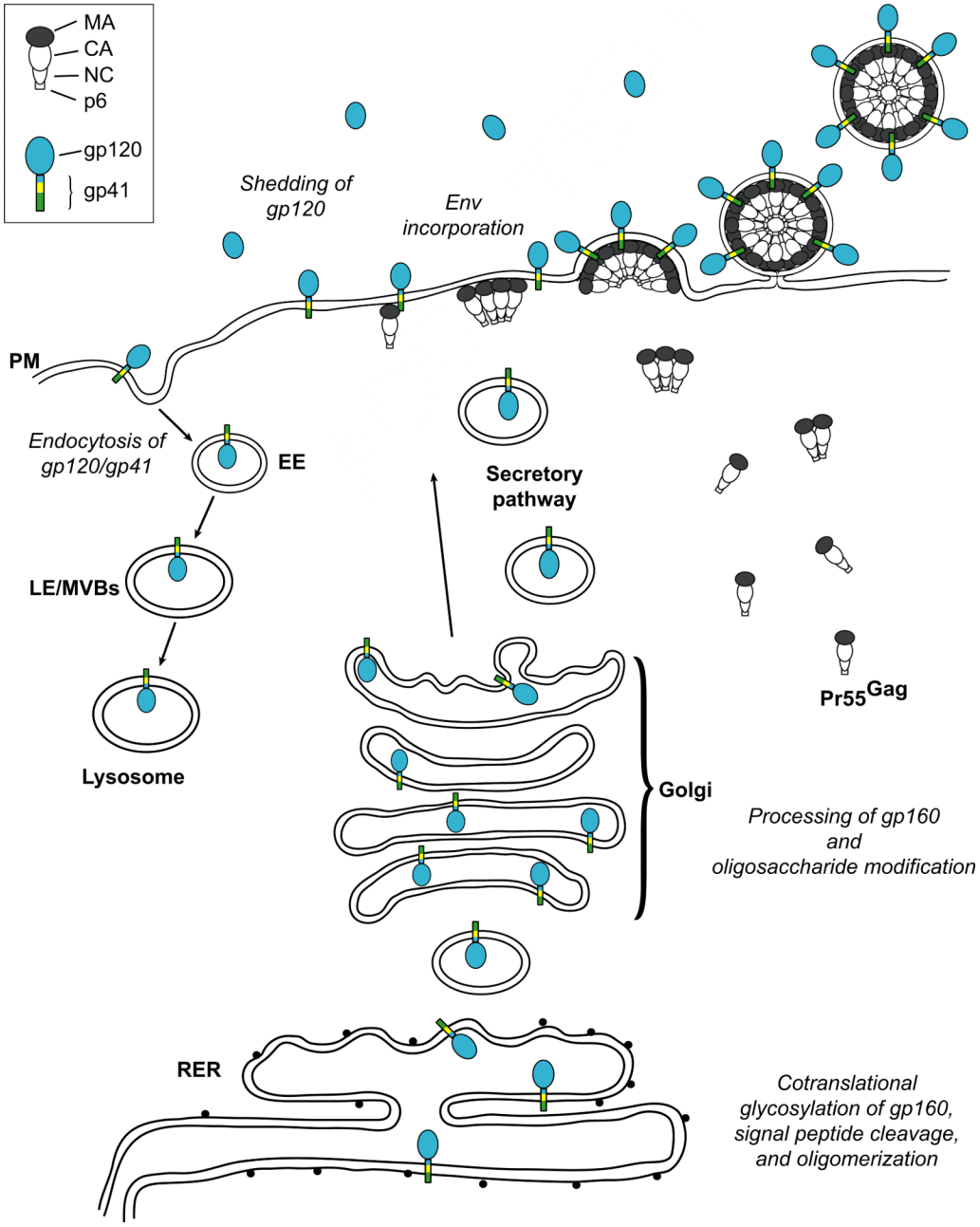

Fig. 1.

HIV-1 Env trafficking. Env is synthesized and glycosylated in the rough endoplasmic reticulum (RER) as a 160 kDa precursor protein (gp160), which oligomerizes predominantly into trimers. Precursor Gag ($Pr55^{Gag}$) is synthesized on cytosolic ribosomes and is directed to the plasma membrane (PM) where it multimerizes in lipid rafts (not shown) to form virus particles. Oligomerized gp160 is transported to the Golgi and *trans*-Golgi network where it is processed to yield mature SU gp120 and TM gp41. Complexes of gp120-gp41 traffic through the secretory pathway to the PM and are incorporated as trimeric spikes into virus particles. At the PM, endocytosis of Env into early endosomes (EE) occurs via interaction with clathrin adaptor complexes. The internalized Env can either traffic through the late endosomes/multivesicular bodies (LE/MVBs) for degradation in the lysosome, or be

returned to the PM through recycling endosomes (not shown). Gag and Env domains are defined in the inset at the top left. Illustration adapted from Ono and Freed[229] with permission from Elsevier.

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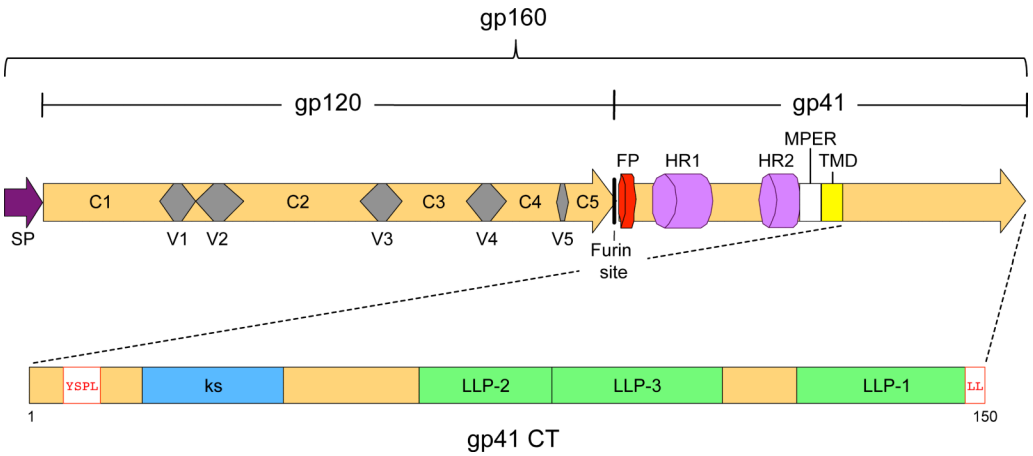

Fig. 2.

Domains of HIV-1 Env. Precursor gp160 contains the signal peptide (SP), which is cleaved during translation. The remaining precursor is cleaved into the surface subunit (gp120) and transmembrane subunit (gp41) in the Golgi complex at the furin site indicated. gp120 contains five variable domains (V1-V5) and five constant domains (C1-C5). gp41 consists of an extracellular domain, containing the fusion peptide (FP), heptad-repeats (HR1 and HR2), and the membrane-proximal external region (MPER), a transmembrane domain (TMD), and a cytoplasmic tail (CT). An enlarged representation of the gp41 CT is shown to highlight several motifs: the internalization signal YSPL, the Kennedy sequence (ks), the amphipathic α-helices LLP-1, -2, -3, and a C-terminal dileucine motif (LL) involved in endocytosis and intracellular distribution of Env.

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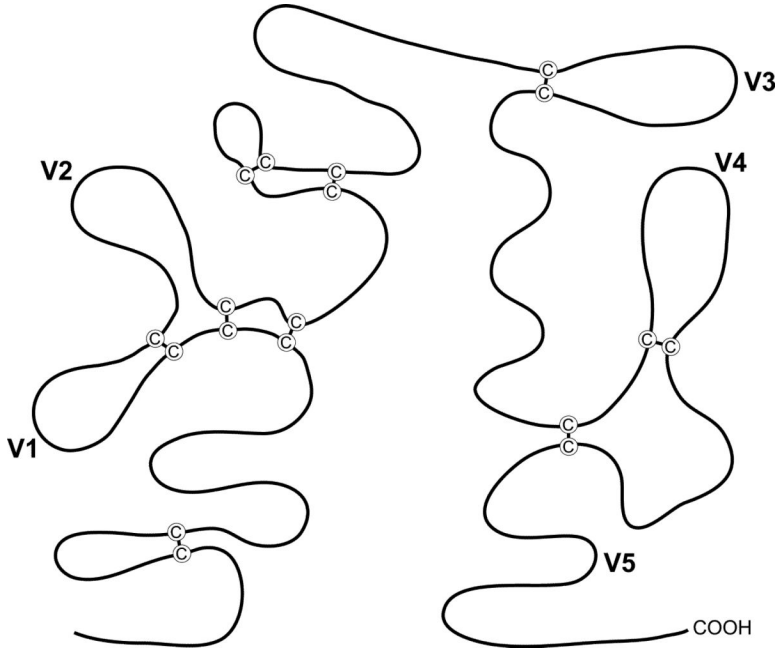

Fig. 3.

Schematic representation of gp120 disulfide bonds. Highly conserved cysteine (C) residues in gp120 form disulfide bridges that are important for Env tertiary structure. Variable domains V1-V5 are indicated. Illustration adapted from Leonard *et al.*[39], originally published in The Journal of Biological Chemistry. © The American Society for Biochemistry and Molecular Biology.

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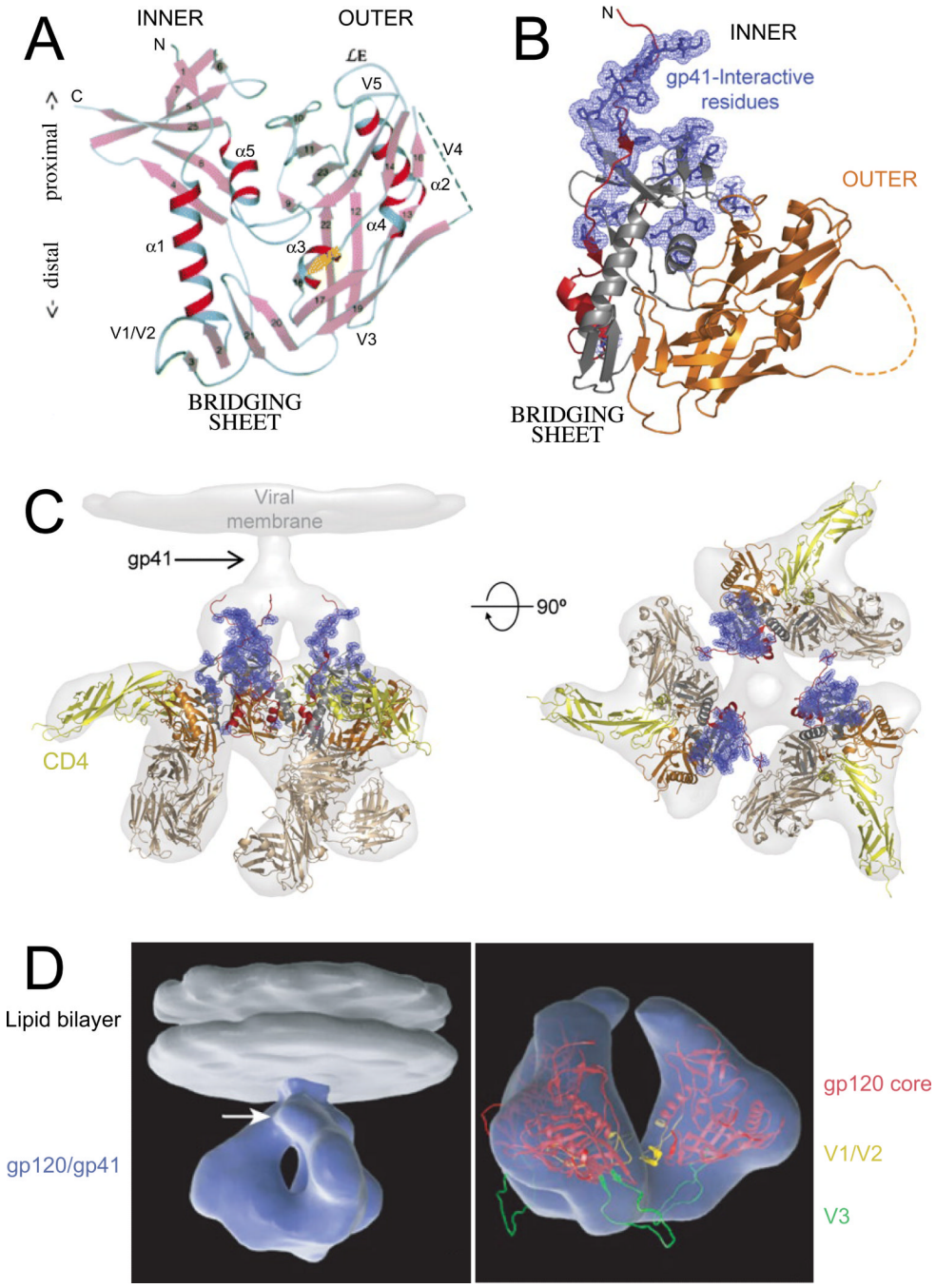

Fig 4.

HIV-1 Env gp120 and gp41 structures. (A) Ribbon diagram of gp120 core containing α-helices (α1-5), β-strands (1-25), with relative positions of variable loops (V1-V5) and N and C-termini shown. The orientation of gp120 in this diagram places the viral membrane toward the top and the cell membrane toward the bottom. When gp120 is bound to CD4 it forms a “bridging sheet” consisting of four β-strands, which separates the inner and outer domains of gp120 relative to their orientation in the trimeric complex. Image reprinted by permission from Macmillan Publishers Ltd: Nature[107], copyright 1998. (B) Ribbon diagram of gp120 core (as in panel A) with N- terminus (red) and gp41 interaction site (blue) shown. The inner domain is shown in red and grey and the outer domain is shown in orange. The

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bridging sheet, which shares elements from both inner and outer domains, is in grey and orange. (C) Trimeric gp120 (same colors as in panel B) bound to three molecules of CD4 (yellow) and Fab from neutralizing antibody 17b (brown), used to stabilize the gp120 structure, superimposed onto the electron density observed by cryoelectron tomography (light grey). Orientation of this structure rotated 90°, which places the viral membrane in the plane of the page, is also shown on the right. Images (panels B, C) reproduced from Pancera *et al.*[113] with permission. (D) Three-dimensional representation of HIV-1 Env in its CD4-bound conformation. (Left) A trimeric Env spike (blue) anchored in the lipid bilayer of the viral membrane (grey) is shown. The white arrow indicates the predicted location of gp41. (Right) Ribbon diagram of gp120 core (red) superimposed on the density map (blue) with V1/V2 loop (yellow) and V3 loop (green) shown. Reprinted by permission from Macmillan Publishers Ltd: Nature[115], copyright 2008.

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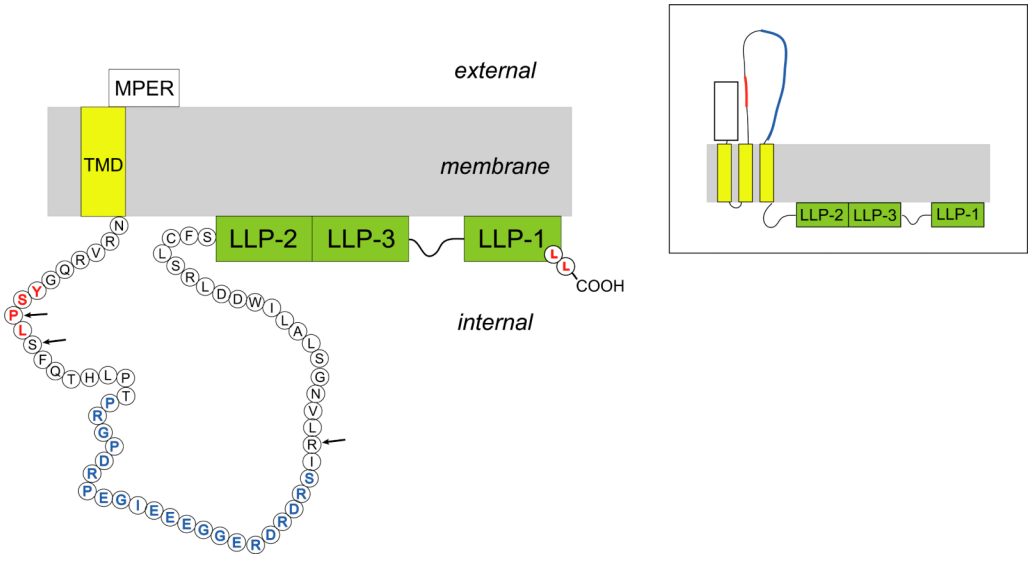

Fig 5.

Models of HIV-1 gp41 CT topology. (Left) Traditional model of the gp41 CT with a single membrane-spanning domain, most likely found in virions. This single-pass model contains the entire CT inside the virion (internal). gp41 domains are the same as indicated in Fig 2. Sites of mutation that confer resistance to AME, which were later shown to become new HIV-1 PR cleavage sites, are indicated (black arrows).[145,146,147] (Right) Alternative three-membrane-spanning topology of gp41 CT, which exposes portions of the gp41 CT to the extracellular space, may also exist with a detectable frequency in HIV-1 Env-expressing cells in addition to the more traditional model. Illustration adapted from Steckbeck *et al*.[144]

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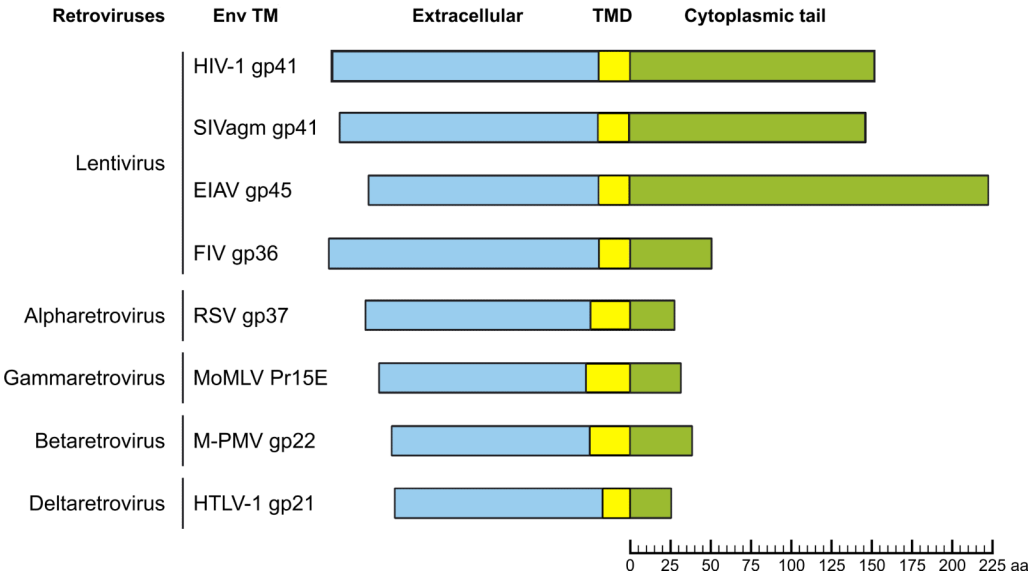

Fig. 6.

Schematic representation of retroviral TM subunits, indicating the lengths [in amino acids (aa)] of their respective cytoplasmic tails. As illustrated, lentiviral TM CTs tend to be significantly longer than those of other retroviruses, with the exception of feline immunodeficiency virus (FIV). SIVagm, simian immunodeficiency virus from African green monkey; MoMLV, Moloney-MLV; HTLV-1, human T-cell lymphotropic virus type 1. Other viral acronyms are defined in the text.

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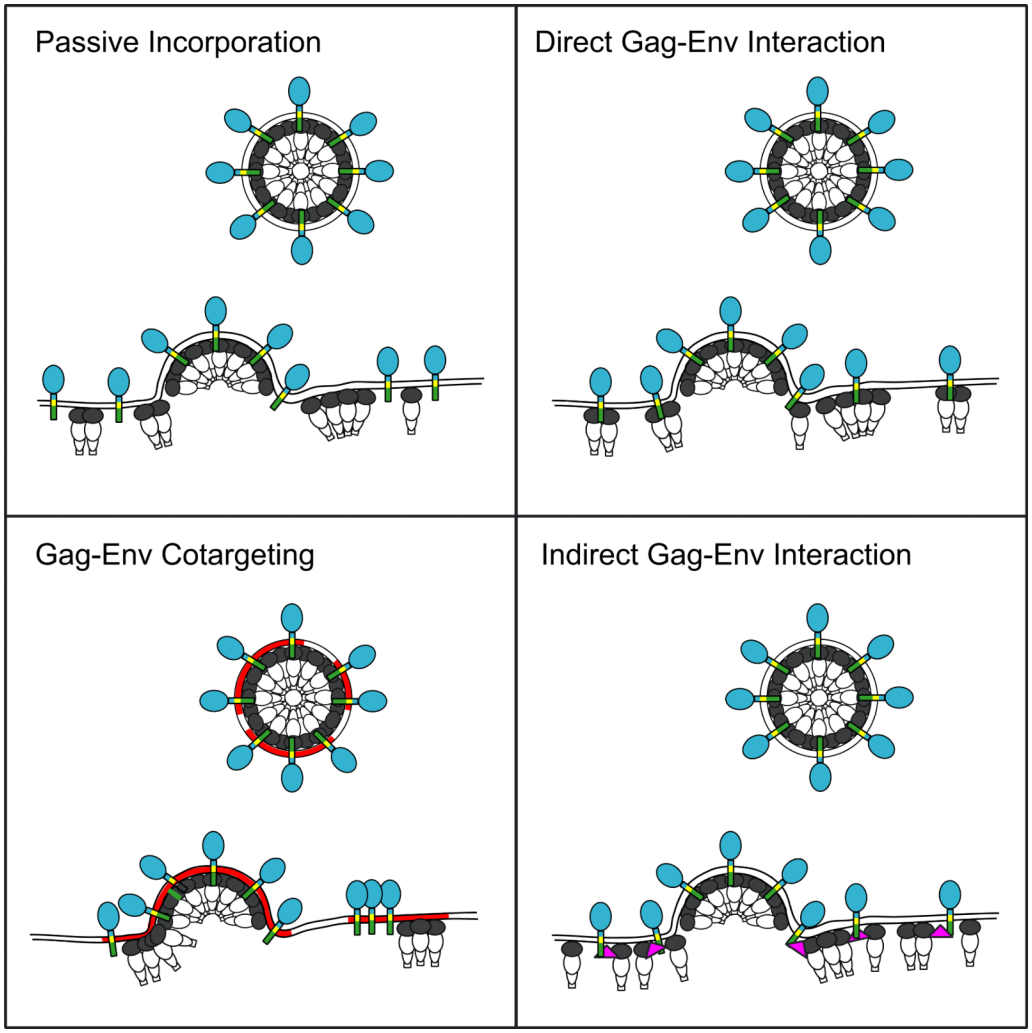

Fig. 7.

Models for HIV-1 Env incorporation. The passive incorporation model (A) postulates no interaction between Gag and Env. The direct Gag-Env interaction model (B) takes into account evidence that the MA domain of Gag interacts with the CT of gp41. The Gag–Env cotargeting model (C) suggests that Env is enriched in virus assembly domains through lipid raft (brown) association. The indirect Gag–Env interaction model (D) provides an alternative explanation for Env incorporation that involves an adaptor protein that binds both Env and Gag. The association of Gag with lipid rafts in all models is assumed but not shown. The color scheme for Gag and Env is the same as in Fig. 1.