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Review Article

Retroviral Env Glycoprotein Trafficking and Incorporation into Virions

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Together with the Gag protein, the Env glycoprotein is a major retroviral structural protein and is essential for forming infectious virus particles. Env is synthesized, processed, and transported to certain microdomains at the plasma membrane and takes advantage of the same host machinery for its trafficking as that used by cellular glycoproteins. Incorporation of Env into progeny virions is probably mediated by the interaction between Env and Gag, in some cases with the additional involvement of certain host factors. Although several general models have been proposed to explain the incorporation of retroviral Env glycoproteins into virions, the actual mechanism for this process is still unclear, partly because structural data on the Env protein cytoplasmic tail is lacking. This paper presents the current understanding of the synthesis, trafficking, and virion incorporation of retroviral Env proteins.

1. Introduction

All replication-competent retroviruses encode genes for three major proteins: Gag, Pol, and Env. Complex retroviruses, such as human immunodeficiency virus type 1 (HIV-1), encode additional regulatory and accessory proteins required for efficient replication in host cell or the infected host organism. Gag, an essential retroviral protein, is necessary and sufficient for the assembly, budding, and release of virus-like particles (VLPs) in all types of retroviruses except the spumaviruses. Gag is synthesized on cytosolic ribosomes and is assembled as a polyprotein precursor. During and/or shortly after budding and release, the polyprotein is cleaved into several domains by the viral protease (Figure 1) as reviewed in [1–3]. The major domains of the precursor Gag are the matrix (MA), capsid (CA), and nucleocapsid (NC). The primary role of the N-terminal MA domain is targeting of the Gag precursor protein to the site of assembly, typically the plasma membrane (PM). In general, electrostatic interactions between basic amino acid residues in MA and the acidic inner leaflet of the PM are important for Gag-membrane targeting [4, 5]. In the case of HIV-1, the N-terminal myristate group and a cluster of basic residues in the MA domain of the HIV-1 Gag that interacts with phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) together target the Gag precursor $Pr55^{Gag}$ to the PM [6, 7]. Although the Gag-membrane targeting of both murine leukemia virus (MLV) and Mason-Pfizer monkey virus (MPMV) is also affected by $PI(4,5)P_2$ modulation [8, 9], it has been reported that the membrane targeting of Rous sarcoma virus (RSV) and human T-lymphotropic virus type 1 (HTLV-1) is largely independent of $PI(4,5)P_2$ [10, 11]. The MA domain also plays a role in the incorporation of the Env glycoprotein into virions. The CA domain is important for Gag-Gag interactions during virus assembly and constitutes the outer part of the viral core after Gag processing by the viral protease [12–14]. NC is the primary nucleic acid binding domain of Gag. This small, basic domain is responsible for the binding and incorporation of the viral RNA genome into virions, which is mediated by Gag interactions with genomic RNA.

Gag proteins are synthesized and transported to the PM. Many studies demonstrate that the major site of HIV-1 assembly is the PM [15–18], although late endosomes could be a platform for virus assembly under specific conditions [19]. In primary macrophages, HIV-1 has been shown to assemble in endosomal vesicles. However, studies

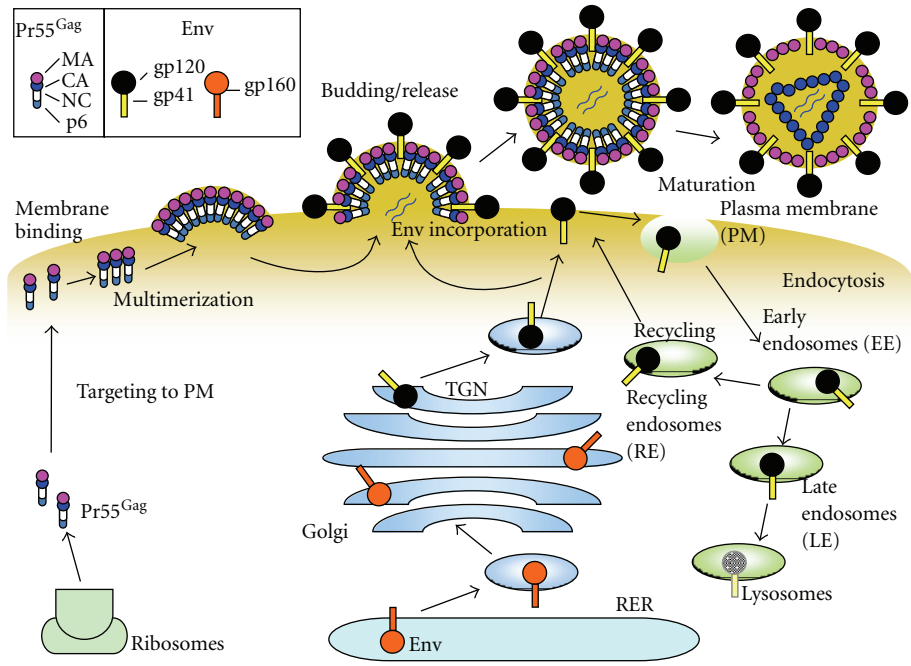


Figure 1: Synthesis and trafficking of HIV-1 Gag and Env proteins. Precursor Gag ($Pr55^{Gag}$) (left) is synthesized on cytosolic ribosomes and traffics to the plasma membrane (PM), where it forms multimers (middle). Env is synthesized as the gp160 precursor, and undergoes glycosylation and oligomerization in the RER. Oligomerized gp160 is transported to the Golgi and the TGN, where it is processed into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular enzymes. The gp120/gp41 complexes are transported through the secretory pathway to the PM and are incorporated into virus particles (middle). At the PM, most of the Env protein is endocytosed into early endosomes (EE), which mature into late endosomes (LE) and then into lysosomes for Env degradation (right). However, some Env proteins are recycled to the PM through recycling endosomes (RE). During and after virus release, processing of $Pr55^{Gag}$ by virus proteases yields mature virions. The protein domains of $Pr55^{Gag}$ and Env are illustrated in the insert at the top left. The illustration was adapted from Checkley et al. with permission from Elsevier [23].

have recently suggested that the above vesicles are not late endosomes but rather membrane invaginations connected to the PM [20–22].

In addition to Gag, the other major structural retroviral protein is the Env glycoprotein. Env proteins are required for virus entry into target cells and are thus essential for forming infectious retroviral particles. In this paper, we discuss current knowledge about the biosynthesis, intracellular trafficking, and virion incorporation of retroviral Env proteins, as well as the membrane microdomains involved in virus assembly and/or transfer. Most of this information was obtained from studies on HIV-1.

2. Env Biosynthesis and Trafficking to the Plasma Membrane

Retroviral Env glycoproteins are synthesized from a spliced form of the viral genomic RNA as reviewed in [23–25] (Figure 1). Translation of the Env protein occurs on ribosomes bound to the endoplasmic reticulum (ER) and starts with the leader sequence, which contains a small, N-terminal hydrophobic signal peptide. The Env protein is cotranslationally inserted into the lumen of the rough ER. In the ER, the leader sequence is removed by cellular signal peptidases. In addition, Env polypeptides are N- and O-glycosylated and subsequently trimmed [26, 27]. The number and location of glycosylated residues varies broadly among retroviruses. The hydrophobic transmembrane (TM) domain prevents Env proteins from being fully released into the lumen of the ER [28, 29]. The amino acid sequence following the TM is referred to as the cytoplasmic tail (CT), which varies from 30 to around 150 residues, depending on the virus. Env proteins are folded and assembled into oligomers in the RER. Retroviral Env proteins form trimers [30–33]. The HIV-1 accessory protein Vpu binds to the CD4 receptor through its cytoplasmic domain and downregulates the receptor by transporting it to the proteasome for degradation, thereby preventing premature interactions between Env and its receptor [34–36].

In the Golgi, cleavage of the retroviral Env precursor occurs at a polybasic (e.g., K/R-X-K/R-R) motif by cellular proteases such as furin or closely related enzymes probably within or near the trans-Golgi network (TGN) [37–43]. For HIV-1, the surface glycoprotein gp120 and the TM glycoprotein gp41, which bind together noncovalently, are both formed from the same precursor protein, gp160. Gp160 processing is essential for the activation of Env fusogenicity and virus infectivity [38, 42, 44–46]. Similarly, cleavage of Env is also essential for membrane fusion and virus infectivity in MLV [39, 47–50], in RSV [51, 52], and in mouse mammary tumor virus (MMTV) [53]. A recent

report showed that cleavage of MLV Env by furin also plays an important role in Env intracellular trafficking and incorporation [54]. Although most retroviral Env proteins including that of HIV-1 are associated with intracellular membranes [55–57], at least part of the gp120/gp41 trimer complex traffics through the secretory pathway to the PM. It has been suggested that AP-1, one of adaptor proteins for clathrin-coated vesicle formation, is involved in the correct sorting of HIV-1 Env from the TGN to the PM, [58, 59]. It has been reported that intracellular CTLA-4-containing secretory granules are involved in the trafficking of HIV-1 Env to the PM although the subsequent trafficking of Env after the Golgi is not well understood [60].

After reaching the PM, like those of other lentiviruses, HIV-1 Env undergoes rapid endocytosis, which is mediated by the interaction between the $\mu 2$ subunit of the clathrin adaptor AP-2 and a membrane-proximal, Tyr-based motif (YxxL) in the gp41 CT [58, 61, 62]. Although some of the endocytosed Env is recycled back to the PM, most retroviral Env is associated with intracellular membranes [63, 64]. The level of gp120-gp41 oligomers on HIV-1 virions is relatively low [33]. Maintaining low levels of Env at the cell surface allows the infected cells to evade the host immune response and to avoid induction of Env-mediated apoptosis. Gammaretroviruses such as MLV and MPMV also have dileucine- and Tyr-based motifs in their Env CT. These motifs are important to regulate intracellular trafficking of Env of both retroviruses via interactions with clathrin adaptors [65, 66].

As for pseudotyping of gammaretroviruses, it has been reported that the feline endogenous retrovirus RD114 Env does not allow pseudotyping with viral cores from lentiviruses such as SIV, whereas the RD114 Env is incorporated into MLV virions [67–69]. Intracellular trafficking of Gag and Env was examined using a set of chimeric viruses between MLV and RD114 [57]. Interestingly, it was found that the RD114 Env was mainly localized along the secretory pathway, whereas the MLV Env was mostly localized in endosomes, and that intracellular localization was dependent on specific motifs in the Env CT [57]. In addition, subsequent work revealed that an acidic cluster in the RD114 Env CT regulates assembly of not only the RD114 Env but also the MLV Env through the interaction with a host factor, phosphofurin acidic-cluster-sorting protein 1 [66].

3. Env Incorporation into Virions

Several models have been proposed for the incorporation of retroviral Env glycoprotein into virions as reviewed in [23, 70] (Figure 2).

3.1. Passive Incorporation. Passive incorporation is the simplest model for the incorporation of Env proteins into virus particles (Figure 2(a)). There are several lines of evidence supporting this model.

First, viral pseudotyping with a foreign glycoprotein can occur easily in many cases although there are some exceptions, one of which is the exclusion of HIV-1 or SIV Env with the long CT from most retrovirus cores [70]. With respect to HIV-1, the virus can be pseudotyped with Env glycoproteins not only from several other retroviruses but also with those from other virus families such as ortho (para) myxoviruses and flaviviruses [71–84].

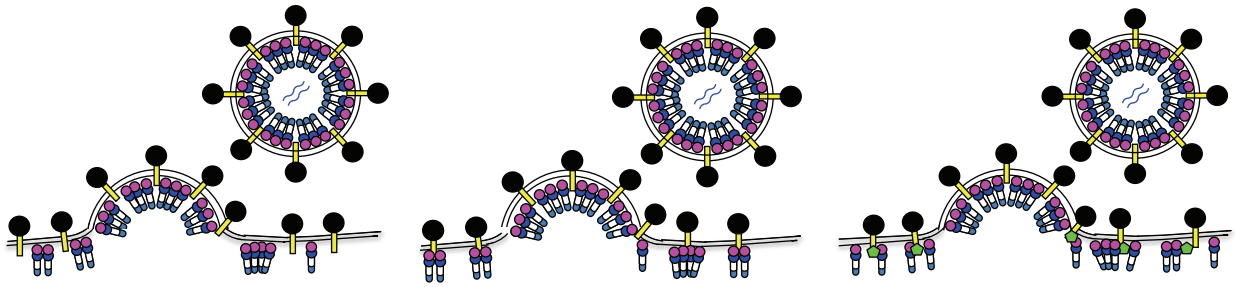
Second, retroviruses allow passive incorporation of host membrane proteins into virus particles [85–87]. Most cellular proteins are incorporated into the retrovirus envelope without significant sorting [88, 89].

Finally, in the case of HIV-1, several studies have demonstrated that the gp41 CT can be removed without affecting incorporation of the Env into virions, although this has been shown to occur only for some laboratory cell lines such as HeLa or 293T [90–94].

3.2. Regulated Incorporation through Direct Gag-Env Interactions. Although several lines of evidence support the passive incorporation model for retroviral Env, there is much evidence indicating that Env incorporation into virions is regulated by direct interactions between Gag and Env proteins (Figure 2(b)). Although removal of the gp41 CT sequence of HIV-1 has little effect on Env incorporation in some cell types, as described above, smaller deletions in CT regions cause severe defects in Env incorporation [95–100]. The MA domain of Gag has been shown to be important for Env incorporation into virions [91, 92, 101, 102]. The defect in Env incorporation caused by deletion of the gp41 CT is reversed by several MA mutations, indicating that an interaction between Env and the MA domain of Gag is required for incorporation of full-length Env into virions, at least in the case of HIV-1 [93, 98].

More evidence for direct Gag-Env interaction comes from the finding that HIV-1 Env directs Gag budding to the basolateral surface of polarized epithelial Madin-Darby canine kidney (MDCK) cells through the CT of HIV-1 Env, whereas Gag alone buds in a nonpolarized fashion [103–106]. The Tyr-based motif in the gp41 CT is also utilized in polarized budding of HIV-1 in lymphocytes [107]. Surprisingly, the polarized budding of HIV-1 in MDCK cells could also be promoted by MLV and HTLV-1 Env through their CT [108]. It also has been reported that coexpression of $Pr55^{Gag}$ inhibits endocytosis of HIV-1 Env through its interaction with the gp41 CT [63]. Another example of the specific Gag-Env interactions was demonstrated using Gag and Env proteins of MLV and HIV-1 in rat neurons [109]. Similarly, MLV Env is preferentially recruited onto MLV Gag through its CT domain in the presence of both MLV and HIV-1 cores although the authors also show an alternative mechanism by which the recruitment to HIV-1 budding sites is independent of the CT domain of MLV Env [110]. Furthermore, RSV Env is exclusively recruited to RSV budding sites through its CT, suggesting that the interaction between Env and Gag is direct in the case of this avian retrovirus [111].

In addition to the circumstantial evidence discussed above, some biochemical data suggest a direct interaction between Gag and Env. *In vitro* binding between MA and a gp41 CT-GST fusion protein has been reported for both



(a) Passive Incorporation

(b) Regulated Incorporation-1 (Direct Gag-Env Interaction)

(c) Regulated Incorporation-2 (Indirect Gag-Env Interaction)

Figure 2: Proposed models for Env incorporation. (a) The passive incorporation model assumes no interaction between Gag and Env. (b) In the first regulated incorporation model, a direct interaction between the MA domain of Gag and the CT domain of Env occurs during Env incorporation. (c) In the second regulated incorporation model, Gag and Env interact indirectly through a bridging protein (green pentagon) that binds to both proteins. The color scheme for Gag and Env is the same as that in Figure 1. The illustration was adapted from Checkley et al. with permission from Elsevier [23].

HIV-1 and SIV [112, 113]. Peptides corresponding to a large central domain of gp41 CT inhibited the capture of membrane-free $Pr55^{Gag}$ with an anti-p24 antibody [114]. In addition, a stable, detergent-resistant gp41-$Pr55^{Gag}$ interaction was detected in immature HIV-1 virions. The retention of gp41 in detergent-treated virions is dependent on the CT region, suggesting a direct or indirect interaction between $Pr55^{Gag}$ and gp41 [115, 116].

3.3. Regulated Incorporation through Indirect Gag-Env Interactions. In the third model, it is assumed that host cellular factors (mostly proteins) play a role in bridging Gag and Env in virus-infected cells (Figure 2(c)). Several host factors have been reported to bind to Gag and/or Env of HIV-1 or SIV however, only a couple of host factors were shown to be required for Env incorporation and/or viral replication.

The 47-kDa tail-interacting protein (TIP47) has been reported to bridge Gag and Env, allowing efficient Env incorporation in HIV-1 [117, 118]. The same group also showed that both the WE motif near the N-terminus of the MA domain and the YW motif in the gp41 CT domain are important for interactions between Gag or Env and TIP47 [118]. In a subsequent paper, the same group showed that mutations in either the WE motif of MA or the YW motif in the gp41 CT caused defects in virus replication in primary monocyte-derived macrophages [119]. Although this finding of an important role for TIP47 in Env incorporation in HIV-1 has received much attention from retrovirologists, no confirmatory data have been published by other researchers in this field.

Human discs large protein (hDlg1) has been reported to interact with the CT of HTLV-1 Env and to colocalize with both Env and Gag in virus-infected cells [120]. Subsequent work demonstrated that Dlg1 also binds HIV-1 Gag and that the expression level of Dlg1 is inversely correlated with HIV-1 Env expression and incorporation levels of the Env proteins, although the mechanism behind this phenomena needs to be investigated [121].

Prenylated Rab acceptor 1 (PRA1), which was identified as a Rab regulatory protein, was reported to be a binding partner for the SIV gp41 CT in a mammalian yeast two-hybrid (Y2H) assay [122]. Although colocalization of PRA1 and SIV Env was observed, changes in the endogenous levels of PRA1 did not affect virus production, Env incorporation, or infectivity of SIV or HIV-1 [123].

A Prohibitin 1/Prohibitin 2 (Phb1/Phb2) heterodimer was identified as a binding partner of the gp41 CT of HIV-1 using human T-cell lines and tandem affinity chromatography [124]. Phb1 and Phb2 are members of the prohibitin superfamily of proteins, which are localized to several cellular compartments such as the mitochondria, nucleus, and the PM [125, 126]. Gp41 CT mutants, in which binding to Phb1/Phb2 is disrupted, could replicate well in permissive cell types such as MT-4, but could not replicate efficiently in nonpermissive H9 cells [124]. Further analysis is necessary to elucidate the mechanism by which these proteins regulate virus replication through interactions with Env.

Luman, a transcription factor that is mainly localized to the ER, was found to interact with the gp41 CT of HIV-1 in a Y2H screen using a cDNA library from human peripheral blood lymphocytes (PBL) [127]. Overexpression of a constitutively active form of this protein reduced the intracellular levels of Gag and Env, leading to a decrease in virus release. The mechanism for this negative effect on virus assembly involves Luman binding to Tat, which decreases Tat-medicated transcription [127].

By using a Y2H screen with human cDNA libraries, p115-RhoGEF, an activator of Rho GTPase, was found to interact with the gp41 CT through its C-terminal regulatory domain [128]. The gp41 mutants that lost the ability to bind p115 showed impaired replication kinetics in T-cell lines such as SupT1, H9, and Jurkat, suggesting that the gp41 CT could

modulate the activity of p115-RhoGEF to support virus replication [128].

In addition to the host factors described above, calmodulin [129–132] and α-catenin [133–135] have been reported to interact with HIV-1 and/or SIV. However, their roles in virus replication, especially with respect to the Env functions of both proteins, have not been clearly elucidated.

4. Membrane Microdomains

Regardless of whether direct or indirect interactions between retroviral Gag and Env proteins are required for Env incorporation into virions, a great deal of experimental evidence suggests that retroviruses assemble and bud from "membrane microdomains." The most well-known microdomains are "lipid raft(s)," which are enriched in cholesterol and sphingolipids [136, 137]. Lipid rafts are widely thought to function as a platform for the assembly of protein complexes and to allow various biological processes such as cellular transport and signal transduction to proceed efficiently [138, 139]. Lipid rafts are reportedly used as assembly platforms or entry scaffolds in the replication of enveloped viruses such as retroviruses [140–146]. The association of Gag/Env with lipid rafts is important for the regulation of Env incorporation and pseudotyping [143, 144, 147, 148]. Evidence that both the HIV-1 $Pr55^{Gag}$ and Env proteins are preferentially localized to lipid rafts comes from biochemical studies as well as direct observations by microscopy [142, 149, 150].

Another membrane microdomain for retrovirus assembly is the "tetraspanin-enriched microdomain (TEM)" [151–154]. Tetraspanins are a superfamily of cell surface proteins that are ubiquitously expressed in mammalian cells. TEMs also act as platforms for signal transduction and immune responses. TEMs have been reported to be involved in the assembly and release of not only HIV-1, but also HTLV-1 and HCV [155]. When both HIV-1 and influenza virus were produced in the same cell, only HIV-1 colocalized with the TEM marker, and its release was inhibited by an anti-CD9 Ab, which led to extensive aggregation of tetraspanins [156]. Analysis of dynamics of both lipid rafts and TEMs by quantitative microscopy has revealed that components of both lipid rafts and TEMs are recruited during viral assembly to create a new microdomain that is different from preexisting membrane microdomains [153, 157].

There have been three recent reports in which both pseudotyping and microdomain issues were discussed. In the first paper, the authors examined HIV-1 assembly under conditions where the Env proteins of HIV-1 and Ebola virus were coexpressed with HIV-1 Gag in the same cell [158]. They found that infectious HIV-1 virions were released with both types of Env proteins. Interestingly, however, the virions contained either HIV-1 Env or Ebola virus glycoprotein (GP), but not both Env proteins within a single virion. These results suggest that HIV-1 Env and Ebola virus GP localized to distinct microdomains on the surface of the same cell [158]. In the second paper, the subcellular localization of Gag and Env proteins was investigated using a combination of three different retroviral Env proteins (RSV Env, MLV Env, or vesicular stomatitis virus (VSV) G) and two different Gag proteins (RSV or HIV-1) [111]. Both VSV-G and MLV Env were redistributed to the virus budding sites when coexpressed with HIV-1 or RSV Gag. In contrast, RSV Env was mostly transported to RSV budding sites. A subsequent paper from the same group showed that the CT of MLV is not required for recruitment of MLV Env to HIV-1 budding sites, suggesting that there are no specific interactions between MLV Env and HIV-1 Gag [110]. Collectively, these results also suggest that retroviral Env glycoproteins are not recruited to preexisting membrane platforms but rather that they are actively recruited to newly formed microdomains on the cell surface [111].

Human retroviruses such as HIV-1 and HTLV-1 spread more efficiently between target T cells by cell-cell infection than by cell-free infection [159, 160]. Sattentau et al. proposed, in analogy to the "immunological synapse", the "virological synapse (VS)" as a point of contact between virus-infected cells and uninfected cells [161, 162]. The molecular mechanisms of retroviral VS formation are as follows. (1) With respect to HIV-1 T-cell VS, initial contact between virus-infected cells and uninfected cells occurs through gp120-CD4 binding. Subsequent interactions between integrins and ICAMs enforce and maintain the stability of these junctions. (2) The gp120-CD4 interaction recruits CD4, coreceptors such as CXCR or CCR5, adhesion molecules, and filamentous actin into the synaptic area. (3) The cellular secretory machinery and microtubule organizing centers (MTOC) are polarized towards the HIV-1 assembly sites at the PM to form the VS. It has been reported that a so-called microsynapse formed by nanotubes between virus-infected cells and uninfected cells is also involved in cell-cell infection of HIV-1 [84, 163]. In cell-cell transfer of HTLV-1-infected cells, an extracellular matrix structure referred to as the "viral biofilm" was proposed as an alternative to the VS [164]. In addition to HIV-1 and HTLV-1, the spread of MLV between fibroblasts also occurs via the VS [165, 166]. It is noteworthy that assembly of MLV is directed towards cell-cell contact sites through the interaction of the CT of MLV Env with Gag [167, 168]. Although the concept of cell-cell infection through the VS is now well appreciated, the detailed molecular mechanism of VS assembly and its relevance to viral spread *in vivo* will require further elucidation through the use of more advanced techniques.

5. Conclusions and Perspectives

Incorporation of Env glycoproteins into virions is crucial for producing infectious retroviral particles. Although this paper has introduced several experimental models for retroviral Env trafficking and/or incorporation, the correct mechanism for this process is still unclear. The following questions must be clearly addressed to not only gain a better understanding of this complex biological process, but also to develop new antiretroviral compounds that target Env incorporation.

(1) What are the structures of the CTs of retroviral Env proteins? The answers for this question will give

useful information on elucidating a role of the Env CTs in the Env trafficking and/or incorporation in virus-infected cells.

(2) What host factor(s) are necessary for the retroviral Env trafficking and/or incorporation into virions?

(3) Where and how Env and Gag proteins of retroviruses are recruited to the assembly sites in order to form infectious virus particles?

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