Mechanisms for Env Glycoprotein Acquisition by Retroviruses

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Abstract

A mandatory step in the formation of an infectious retroviral particle is the acquisition of its envelope glycoprotein (Env). This step invariably occurs by Env positioning itself in the host membrane at the location of viral budding and being incorporated along with the host membrane into the viral particle. In some ways, this step of the viral life cycle would appear to be imprecise. There is no specific sequence in Env or in the retroviral structural protein, Gag, that is inherently required for the production of an infectious Env-containing particle. Additionally, Env-defective proviruses can efficiently produce infectious particles with any of a number of foreign retroviral Env glycoproteins or even glycoproteins from unrelated viral families, a process termed pseudotyping. However, mounting evidence suggests that Env incorporation is neither passive nor random. Rather, several redundant mechanisms appear to contribute to the carefully controlled process of Env acquisition, many of which are apparently used by a wide variety of enveloped viruses. This review presents and discusses the evidence for these different mechanisms contributing to incorporation.

Introduction

WHILE ALL RETROVIRAL GENOMES encode the three major proteins Gag, Pol, and Env, some complex retroviruses encode additional proteins. For instance, HIV-1 also encodes the proteins, Vif, Vpr, Tat, Rev, Nef, and Vpu. For all retroviruses except the spumaretroviruses, Gag is the only viral protein required to assemble virus-like particles (VLPs) that bud through a host-derived membrane to exit the cell. Gag is expressed and assembled as a polyprotein, which is cleaved into multiple domains by a virus-derived protease during or shortly after budding (Fig. 1). The major domains of Gag are (from N- to C- terminus) matrix (MA), capsid (CA), and nucleocapsid (NC). MA is believed to be the primary membrane-targeting domain of Gag. Both N-terminal fatty acid modification of MA and patches of charged amino acids contribute to membrane binding.¹ CA is the primary structural domain, which forms a curved lattice of tightly packed CA hexamers.^{2–4} NC is the nucleic acid binding domain of Gag and is responsible for binding and incorporating the viral RNA genome. Retroviral assembly essentially comes about through a combination of protein–protein, protein–membrane, and protein–nucleic acid interactions. Each of these interactions can be studied individually, but each can also be enhanced through cooperativity. For instance, CA hexamers can form *de novo* at high concentrations, but this multi-

merization is greatly enhanced by NC–nucleic acid interactions.⁵ In the cell, it is not entirely clear which interaction initiates the assembly process and in what order they proceed.

Gag proteins are all produced in the cytoplasm of cells and bud through a cellular membrane to form the enveloped virus particles. For HIV-1, it is still debated where assembly is initiated, but most evidence suggests that the entire assembly process occurs on the plasma membrane.^{6,7} The most compelling evidence supporting this conclusion comes from imaging studies in living cells using fluorescently labeled Gag where the entire assembly process is viewed on the plasma membrane.^{8,9} It has often been suggested that the site of HIV-1 assembly is cell-type dependent. In macrophages, for instance, assembling VLPs have often been viewed in what appears to be internal endosomal vesicles.^{10,11} However, recent studies have demonstrated that these vesicles are, in fact, elaborate membrane invaginations that are continuous with the plasma membrane.^{12,13} In contrast to HIV-1, betaretroviruses such as Mason–Pfizer monkey virus (MPMV) assemble in the apparent absence of membrane near the microtubule organizing center before trafficking to and budding through the plasma membrane.^{14,15} With other retroviruses the location of viral assembly initiation has not been studied as extensively.

The question of how HIV-1 Gag specifically targets the plasma membrane, which represents a minority of all

FIG. 1. Schematic of HIV-1 genome and genes. Matrix (MA), capsid (CA), nucleocapsid (NC), leader peptide (L), surface protein (SU), transmembrane protein (TM).

membranes in the cell, has received considerable attention in recent years. One proposed mechanism is that this targeting occurs through binding of HIV-1 MA to phosphatidylinositol-(4,5)-biphosphate [PI(4,5)P₂],^{16,17} a lipid found predominantly at the plasma membrane. Purified HIV-1 MA is reported to have a specific binding pocket for PI(4,5)P₂.¹⁸ The functional significance of this pocket is suggested by the observation that destroying the PI(4,5)P₂ in cells, by overexpression of a PI(4,5)P₂-specific phosphatase, greatly reduces HIV-1 assembly and budding at the plasma membrane.^{16,17} Murine leukemia virus (MLV) and MPMV budding have also been reported to be altered by PI(4,5)P₂ modulation.^{19,20} However, distinguishing primary from secondary effects of such experiments is challenging. In addition, all retroviral MA domains have clustered basic residues on one surface,¹ and electrostatic attraction of MA to the acidic inner leaflet of the plasma membrane also plays a role in MA-membrane binding,^{21,22} as it does for numerous cellular proteins. The negative charge on the inner leaflet of the plasma membrane is contributed largely by phosphatidylserine and PI(4,5)P₂. Thus, destroying the latter also affects electrostatics. For retroviral Gag-membrane targeting in general, it remains to be established what the relative contributions are of a possible specific PI(4,5)P₂-binding pocket in MA (as found in HIV-1) and of simple electrostatic interactions.

The Env proteins of all retroviruses are type-1 membrane glycoproteins (Fig. 1). All have a signal peptide that is cleaved during translation in the endoplasmic reticulum (ER). Env glycoproteins are generally believed to form trimers in the ER. After exiting the ER, the glycoproteins are transported through the Golgi where they are variably glycosylated. The Env proteins are all cleaved by furin or a furin-like protease into surface (SU) and transmembrane (TM) subunits, which remain associated with each other. Most retroviral Env proteins traffic to the plasma membrane but are rapidly recycled such that at steady state they are found to be predominantly associated with intracellular membranes. To be incorporated into VLPs, the Env proteins must translocate to the membrane through which retroviral budding occurs and to the budding site. The specific mechanisms for how Env recognizes and traffics to these locations are poorly understood.

Passive Incorporation

Passive incorporation is the simplest explanation for how retroviruses acquire their surface glycoproteins. If Env is expressed at high levels on the membrane through which budding occurs, a degree of Env incorporation will occur through random diffusion to viral budding sites. The simplest evidence that supports this notion is the fact that viral pseudotyping (assembly with a foreign viral glycoprotein) occurs fairly readily.²³ For example, HIV-1 can form infectious particles with Env glycoproteins from most if not all of the retroviral subfamilies including alpharetroviruses,²⁴ betaretroviruses,²⁵ gammaretroviruses,²⁶ deltaretroviruses,²⁷ spumaretroviruses,²⁸ and other lentiviruses.²⁹ HIV-1 can also be pseudotyped with glycoproteins from other viral families, such as rhabdoviruses,³⁰ filoviruses,³¹ arenaviruses,³² togaviruses,³³ coronaviruses,³⁴ paramyxoviruses,³⁵ flaviviruses,³⁶ orthomyxoviruses,³¹ and even insect baculoviruses.³⁷ Even more surprising, HIV-1 often produces a considerably higher titer of infectious particles with certain foreign glycoproteins such as VSV-G than it produces with its native Env glycoprotein. The fact that HIV-1 assembly can occur with a wide breadth of unrelated viral glycoproteins suggests that either all compatible viruses utilize the same mechanism to achieve glycoprotein incorporation or there is no specific mechanism required for Env incorporation.

Analyses of cellular proteins incorporated into VLPs also lend support to passive incorporation. Studies that have examined the composition of host cellular proteins incorporated into HIV-1 and MLV particles produced using an overexpression system found minimal exclusion of host proteins from VLPs.^{38,39} Later studies that sought to catalog the proteins incorporated into HIV-1 particles also found an abundance of host proteins incorporated into viral particles.⁴⁰

Finally, passive incorporation is also supported by the finding that the MA domain of Gag is not strictly required for Env incorporation or infectivity. The MA domain of HIV-1 Gag can be replaced by nonviral membrane-binding protein domains without the virus losing its ability to form infectious particles with certain viral glycoproteins.^{41–43} Because MA is believed to be the targeting domain of Gag, and is the only domain likely to interact with a transmembrane glycoprotein either directly or indirectly, the observation that MA is not strictly required for glycoprotein incorporation severely restricts the notion of specific recruitment to viral budding sites.

Lessons from Exceptions

Generally, viral glycoproteins are enriched in VLPs and cellular proteins are not. Still, plenty of exceptions to this rule exist. An early noted exception is the cellular glycoprotein CD4. Although CD4 is not usually incorporated into retroviral particles, it is efficiently and selectively incorporated into Rous sarcoma virus (RSV) particles when expressed in quail cells.⁴⁴ CD4, like most cellular surface proteins, interacts with networks of other host proteins in the cytoplasm. It has been hypothesized that these interactions can anchor host proteins to the cytoplasm, preventing their accumulation in VLPs.⁴⁴ However, when introduced into a nonmammalian cell type, CD4 could behave more like a viral glycoprotein without binding partners that prevent incorporation. When considering mechanisms of glycopro-

tein incorporation, this observed exception illustrates that some mechanisms may modulate not only inclusion, but also exclusion from VLPs.

Another extensively studied exception is the exclusion of HIV-1 Env from most types of retroviruses. Unlike most retroviral Env proteins, which have short 30–50 amino acid cytoplasmic tails, lentiviral Env proteins, such as HIV-1 Env, have long cytoplasmic tails of 150 amino acids or more. The lentiviral Env proteins are generally able to form infectious particles with only their native Gag or Gag from closely related viruses. HIV-1 Env is also prevented from being incorporated into HIV-1 particles that lack their native MA domain^{41,42} and even into viruses with certain discrete point mutations in their MA domain.^{45–47} Interestingly, modest truncations from the C-terminus of HIV-1 Env generally reduce Env incorporation and viral infectivity.^{48–51} However, large truncations removing most or all of the HIV-1 Env cytoplasmic tail result in a protein that can form infectious particles in certain situations with wild-type HIV-1 Gag,^{52–54} HIV-1 Gag mutants that were incompatible with the full length HIV-1 Env,^{42,45,55} and even Gag from other retroviral families.^{56,57} Similarly, SIVagm Env and RSV Env are also rendered more compatible with foreign retroviruses if their cytoplasmic tails are truncated.^{24,58} It has been proposed that Env cytoplasmic tails can cause steric hindrance, which prevents incorporation into VLPs, unless the Env cytoplasmic tails and MA are matched.^{56,57} These examples as well as the example with human CD4 suggest that all glycoproteins on the plasma membrane can be incorporated into retroviral VLPs (perhaps passively) unless restricted through some sort of dominant interference.

Two additional examples of Gag/Env incompatibility come from the Env proteins of gammaretroviruses RD114 and Gibbon ape leukemia virus (GaLV). Simian immunodeficiency virus (SIV) and HIV-1 have been reported to be incompatible with RD114 and GaLV Env, respectively.^{32,59,60} These combinations stand out because most gammaretroviral Env proteins are compatible with lentiviruses. Taking advantage of the similarity between these proteins and the lentivirus-compatible Env from MLV, chimeras were engineered to pinpoint the sequences responsible for the incompatibility. In both cases, the incompatibility was mapped to the C-terminal 35 amino acid cytoplasmic tail of Env.^{32,59} With RD114 Env, the cytoplasmic tail appears to alter the trafficking of Env, making it inaccessible to SIV.^{59,61,62} With GaLV Env, the glycoprotein cytoplasmic tail is modulated by sensitivity to the HIV-1 accessory protein Vpu.^{63,64} Deletion of Vpu abolishes the incompatibility between these proteins. Both cases support the generalization that all viral glycoproteins are compatible with all enveloped viruses, as long as they traffic to the same cellular membrane, unless something prevents their incorporation into VLPs.

Evidence for Specificity

Despite data suggesting that glycoprotein acquisition is a passive phenomenon, there are several notable counterexamples. For example, when polarized monolayers of Madin-Darby canine kidney (MDCK) cells are infected with HIV-1, viral particles are released predominantly from the basolateral surface, and this polarized viral release is dependent on Env.^{65–68} In the absence of Env, Gag is released equally from

apical and basolateral surfaces. Amazingly, the polarized budding of HIV-1 could also be imparted by MLV Env and human T cell leukemia virus 1 (HTLV-1) Env.⁶⁵ In all cases, the basolateral targeting capability of Env is dictated by the cytoplasmic tail. These findings are based on studies performed in canine epithelial cells with extremely low levels of viral output that was measured primarily using a sensitive ELISA assay for p24. Notably, similar studies using alternative polarized models have not found the same basolateral release of infectious viral particles.^{69,70} It also has been demonstrated that HIV-1 Env cannot direct the budding of Gag to any membrane in the cell, as retention of HIV-1 Env in the ER does not redirect budding away from the plasma membrane.⁷¹

Two analogous examples show that the cellular distribution of Gag can be modulated by Env. The first example comes from work with rat neuronal cells where HIV-1 Env and MLV Env expression cause the relocation of HIV-1 Gag or MLV Gag, respectively, from the axons to the somatic region of the cell.⁷² Unlike the example in polarized MDCK cells, this redistribution occurs only among homologous pairs; MLV Env does not redistribute HIV Gag and HIV Env does not redistribute MLV Gag. More recently, a similar phenomenon has been observed where MLV assembly is directed toward sites of cell–cell contact.⁷³ While MLV Env is independently targeted to sites of cell–cell contact, MLV Gag targeting is Env dependent. This targeted assembly was further shown to be dependent on the cytoplasmic tail of MLV Env (Fig. 2A and B).

A final line of evidence for specificity of Env incorporation comes from imaging studies that visualize the distribution of glycoproteins on the surface of cells by scanning electron microscopy. These studies illustrate that viral glycoproteins appear relatively randomly distributed on the surface of cells when expressed alone, but are robustly recruited to viral budding sites of compatible structural proteins (Fig. 2C–F).^{63,74,75} For instance, when MLV Env is expressed along with HIV-1 Gag, the surface MLV Env is tightly clustered at HIV-1 budding sites and is essentially absent from the rest of the plasma membrane. Surprisingly, this enrichment of MLV Env to HIV-1 budding sites is not dependent on the Env cytoplasmic tail (Fig. 2G and H).⁷⁵

Mechanisms of Specificity

From the experiments described above, at least in some circumstances Env acquisition by retroviruses is an active and specific process, but this information does not identify the mechanism used for incorporation. This section reviews the evidence for three types of interaction that could contribute to specificity: direct interaction among viral proteins, indirect interaction and/or convergent trafficking, and lipid microdomains as assembly platforms.

Direct interaction

At least among lentiviruses, several lines of evidence suggest a direct interaction between the cytoplasmic tail of Env and the MA domain of Gag. First, direct *in vitro* binding between MA and the C-terminal domain (CTD) of Env has been demonstrated for both HIV-1 and SIV.^{76,77} Second, a number of mutations have been characterized in both MA and Env that prevent incorporation of HIV-1 Env into HIV-1 particles

FIG. 2. (A, B) Merged time lapse video imaging the localization of murine leukemia virus (MLV) Gag (red) relative to locations of cell-cell contact (green). Cells expressed (A) wild-type MLV Env or (B) MLV Env $\Delta 16$ C-terminal amino acids (image adapted from Jin *et al.*⁷³). (C, E, G) Secondary electron images illustrating the topology of the cell surface. (D, F, H) Backscatter images illustrating the distribution of the MLV Env on the cell surface. (E–H) Express late domain defective HIV-1 Gag; (C–F) express wild-type MLV Env; (G, H) express MLV Env $\Delta 33$ C-terminal amino acids. (C–H) Scale bars 200 nm.

(discussed in “Lessons from Exceptions”). Importantly, in one example a deletion in HIV-1 Env blocks incorporation into particles, but compensatory mutations in the MA domain of HIV-1 Gag restore incorporation.⁷⁸ Third, as with many viral glycoproteins, HIV-1 Env is prevented from becoming fully fusogenic prior to viral maturation.⁷⁹ With betaretroviruses and gammaretroviruses, fusogenic activity is enabled by cleavage of the C-terminal R-peptide by the viral protease.^{80,81} HIV-1 Env is not cleaved by protease; yet, the fusogenicity of Env is dictated by the protease processing of HIV-1 Gag,⁷⁹ suggesting that a direct interaction between Gag and Env in the immature particle maintains Env in a nonfusogenic form. In summary, for retroviruses there is little doubt that at least in some instances, direct interactions occur between Gag proteins and their native Env glycoprotein. This conclusion is consistent with knowledge about other enveloped viruses, such as alphaviruses, for which direct interactions between structural and transmembrane proteins are required for assembly.⁸² However, it is unlikely that these interactions alone promote incorporation into retroviruses, and it is unlikely that

direct interactions play any role among heterologous Gag–Env combinations.

Protein intermediates/convergent trafficking

Independent of direct interactions, both Gag and Env interact with cellular trafficking machinery that has the potential to deliver both proteins to the same microenvironment for assembly, or even to serve as a bridge between the two types of proteins. The most extensively studied interaction motif, which is found in many if not most Env proteins, is the membrane proximal YxxL motif.⁸³ This sequence interacts with the cellular trafficking adapter protein complex 2 (AP-2).^{84–86} Mutation of this motif in HIV-1 Env blocks AP-2 binding,^{85,86} reduces the rate of Env endocytosis,⁸⁷ eliminates polarized budding in MDCK cells,⁶⁷ and reduces viral replication in certain situations.⁸⁸ Most lentiviral Env proteins have a conserved YxxL near their transmembrane domain. Some nonlentiviruses have this conserved feature as well, but not necessarily in the same place. For instance, all gammare-

troviral Env proteins also have a YxxL motif, but further from the membrane-spanning domain than in lentiviral Envs. Nonetheless, mutation of this motif in MLV Env can alter protein distribution in the cell⁸⁹ and abolish polarized budding of HIV-1 Gag in MDCK cells.⁶⁵ At the very C-terminus of the HIV Env CTD is a conserved dileucine motif that binds to the adapter protein complex 1 (AP-1) and may contribute to Env trafficking.^{90,91} Mutation of this motif eliminates AP-1 binding and alters the intracellular distribution of Env. Because HIV-1 Gag has also been reported to interact with various AP complexes,^{92,93} it is conceivable that this type of complex serves as a transient intermediate between Gag and Env.

A peculiar trafficking phenotype is observed with the gammaretrovirus Env RD114. As mentioned above, the cytoplasmic tail of RD114 causes Env proteins to become incompatible with the lentivirus SIV but compatible with the gammaretrovirus MLV.⁵⁹ This loss of compatibility correlates with a loss of Env association with late endosomes. MLV Env partially associates with late endosomes and is associated with MLV and SIV Gags at these compartments. In contrast, RD114 Env by itself does not associate with these compartments but is relocalized to them in the presence of MLV Gag, but not SIV Gag. Both Env proteins display a mixture of furin processed and unprocessed proteins on the cell surface, but only fully processed Env is incorporated into VLPs. These experiments have been interpreted to indicate that retroviral assembly occurs, or at least commences, at these late endosome compartments, and that the incompatibility with SIV occurs because Env does not reach this location. Further dissection of the RD114 cytoplasmic tail revealed that a cluster of acidic amino acids at the C-terminus is responsible both for the loss of association with late endosomes and the loss of infectivity.⁶² The cellular protein PACS-1 is implicated in binding to this acidic cluster in RD114 and causing retrograde transport to the trans-Golgi network. Knockdown of PACS-1 partially relieves the RD114 Env incompatibility with SIV Gag. However, since viral particles can appear to be associated with late endosomes as a result of being endocytosed by the cell after budding,^{43,94} these data do not necessarily prove that assembly occurs at late endosomes. It is nonetheless intriguing that alterations in Env trafficking can affect incorporation and infectivity in a virus-specific manner.

It is almost universally agreed that furin cleavage of Env, to create the mature SU and TM proteins, is required for fusogenic activity and viral infectivity.^{75,95–98} At least in some cases, furin cleavage is also required for incorporation into viral particles.^{99,100} Still, whether lack of incorporation is due to the protein being unable to reach the cell surface is under dispute. Several studies have observed unprocessed Env expressed on the cell surface but only processed Env in viral particles.^{100,101} In fact, a point mutation in MLV Env that disrupts furin cleavage results in a protein that is abundant and clustered on the cell surface, but is no longer enriched at HIV-1 budding sites.¹⁰² This finding is particularly telling since the cytoplasmic tail of this protein is identical to that of wild-type MLV Env. Thus, the incorporation of Env proteins into viral particles appears to involve multiple interactions, and cannot simply be the result of random incorporation or direct interaction.

Other cellular proteins that have been reported to interact with the HIV-1 Env cytoplasmic tail include tail interacting

protein 47 (TIP47),¹⁰³ the prenylated Rab acceptor,¹⁰⁴ and p115-RhoGEF.¹⁰⁵ TIP47, in particular, has been proposed to be a physical connector between the cytoplasmic domain of HIV-1 Env and the MA domain of HIV-1 Gag. Depletion of this cellular protein by RNAi reduces HIV-1 Env incorporation into HIV-1 particles and viral infectivity but does not affect the infectivity of HIV-1 particles pseudotyped with VSV-G.¹⁰³

Membrane microdomains

Many lines of evidence suggest that retroviruses bud from membrane microdomains with properties consistent with lipid rafts.^{6,7,106,107} Because many viral glycoproteins also associate with these domains, it has been proposed that lipid raft association is one factor that leads to selective Env incorporation and pseudotyping.^{108–112} However, HIV-1 Gag and Env have also been shown to associate with tetraspanin-enriched microdomains (TEMs),^{113–115} which are distinct from membrane rafts.¹¹⁶ Interestingly, the hemagglutinin (HA) protein of influenza virus, which strongly associates with membrane rafts, does not colocalize with TEMs.^{113,117} When HIV-1 and influenza are produced in the same cell, the two viruses appear at distinct locations on the surface, but only the HIV-1 particles are found to colocalize with TEM markers.¹¹⁷ An analysis of the dynamics of both TEM and raft markers relative to viral budding sites revealed that both raft and TEM markers associate with HIV-1 budding sites, and the mobility of TEM (and not raft) markers is altered by HIV-1 Gag.¹¹⁵ As TEM and raft markers generally do not cosegregate in cells and as the two have distinct properties, these data may suggest that retroviral assembly leads to the creation of a lipid microdomain distinct from any preexisting microdomain and is favored by many viral glycoproteins.

An interesting finding that further confuses the notion of assembly at microdomains comes from experiments in which cells express HIV-1 Gag along with two different types of viral glycoproteins, HIV-1 Env and Ebola glycoprotein. Infectious HIV-1 particles are produced with both types of glycoprotein, but the assembly is quantal with each particle containing either one type of glycoprotein or the other, but not both.¹¹⁸ These data are interpreted as evidence that HIV-1 assembly occurs quantally on a single lipid raft that contains a single type of viral glycoprotein. However, these data lack an explanation for how the different kinds of glycoproteins segregate themselves into distinct microdomains prior to assembly.

Conclusions

In summary, the following generalizations can be made about the factors that modulate viral glycoprotein incorporation into retroviral particles. First, incorporation requires that Env traffics to the membrane of the same subcellular compartment through which budding occurs. Viral glycoproteins are entirely dependent on cellular machinery for transportation within the cell, which means that either Env has to be delivered to the correct membrane where assembly occurs or Gag has to be recruited to assemble at the membrane where Env resides. Both scenarios probably occur under certain circumstances. Alterations in the virus or the cell that change the localization of either of these proteins have the potential to abolish incorporation. Second, on reaching the appropriate membrane, a combination of factors causes viral

glycoproteins to be recruited to or excluded from the budding virus. Recruitment to retroviral particles could occur from direct interaction among viral proteins or from indirect interactions through cellular proteins, lipid microdomains, or other factors that are yet to be elucidated. Exclusion could occur through steric hindrance or through interactions that prevent incorporation into particles. The final extent of Env inclusion into a retrovirus can be thought of as a summation of all of these forces. The resulting complexity of the process confounds experiments to test the contribution of any one force. With a clearer understanding of the various forces that contribute to this elegant process, the mechanism used by each type of virus will become easier to discern.

Acknowledgments

I thank M.M. Kroll, V.M. Vogt, T.M. Lucas, S.L. Liu, D. Gregory, and S. Janaka for helpful comments. SEM studies were performed at the University of Missouri Electron Microscopy Core facility. This research was supported by U.S. Public Health Service Grant AI73098.

Author Disclosure Statement

No competing financial interests exist.

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