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HIV-1 Assembly at the Plasma Membrane

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Introduction

Trafficking of virus components to proper intracellular locations where virus assembly occurs is a major aspect in a virus life cycle. Improper localization of viral proteins and genomes would either disrupt production of infectious progeny virions or block efficient transmission to other host cells. Therefore, mechanisms ensuring proper trafficking of viral components and efficient assembly of these components at the right place may serve as targets for antiviral strategies. Nevertheless, although some efforts are underway, antivirals inhibiting virus assembly in general and those targeting trafficking of viral components in particular have yet to be developed. In case of HIV-1, this is chiefly because the nature of factors involved in trafficking and localization of viral components are poorly understood. In this manuscript, I will summarize our current knowledge on mechanisms that determine the sites of HIV-1 assembly with a major focus on the role of a plasma membrane phospholipids, phosphatidylinositol-(4,5)-bisphosphate.

HIV-1 Assembly and Release

Assembly of retrovirus including HIV-1 is driven by a viral structural protein known as Gag [1]. This process consists of multiple steps including: 1) Gag targeting to the site of assembly, 2) Gag interaction with lipid bilayer membrane, 3) multimerization of Gag, 4) genomic RNA encapsidation, 5) incorporation of Env into virus particles, and 6) budding and release of nascent particles. In the case of HIV-1, Gag is synthesized as a precursor polypeptide Pr55^{Gag}, which consists of four major structural domains, MA, CA, NC, and p6, and two spacer peptides, SP1 and SP2 [1] (Fig. 1). These structural domains within Pr55^{Gag} correspond to mature Gag proteins formed by viral-protease-mediated processing of Pr55^{Gag}, which occurs concomitantly or immediately after virus particle release. In addition to structural domains, locations of Gag regions involved in each of assembly steps are now well documented thanks to extensive mutagenesis studies [1]. For example, determinants for Gag targeting, membrane binding, and Env incorporation are all present in MA, whereas efficient Gag multimerization requires both CA and NC. CA promotes Gag-Gag interaction through its dimerization, whereas RNA bound to NC is thought to serve as scaffolding. Specific encapsidation of viral genomic RNA is mediated by NC zinc finger domains, while p6 contains peptide sequences that recruit cellular protein complexes essential for efficient virus particle release. The order of steps in virus assembly process and interdependence between them remains to be determined.

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Plasma Membrane as the Major Site of HIV-1 Assembly

It has been long known that in T cells and other tissue culture cell lines such as HeLa, HIV-1 assembly takes place primarily at the plasma membrane (PM). However, early EM studies suggested that this is not the case for primary monocyte-derived macrophages (MDMs). In MDMs, Gag and virus particles were observed to accumulate in apparently intracellular compartments [2–5]. Recent studies suggested, however, that at least a subset of these intracellular compartments, now known as Virus-Containing Compartments (VCC), is connected to the PM and therefore can be regarded as a subdomain of the PM [6–8]. Indeed, at earlier time points after transfection of a Gag-encoding plasmid, even in MDMs, Gag was predominantly found at the cell surface [9]. Altogether, these studies support that the PM (or its invaginated subdomain) is the primary site of virus assembly in tissue culture cell lines as well as T cells and macrophages that are natural target of HIV-1. It remains to be determined how Gag is transported through the cytoplasm before reaching the PM. Readers interested in relationships between intracellular trafficking of Gag and HIV-1 assembly are referred to recent reviews that discuss this topic extensively [10–13].

MA mediates Gag localization to the PM

As mentioned earlier, MA contains a determinant for Gag localization to the PM. Gag proteins containing large deletions in MA have been observed to localize either specifically to the ER that is the most abundant membranous compartment [14] or promiscuously to intracellular and plasma membranes [15]. Studies of MA amino acid substitution mutants further identified that a highly basic sequence spanning from residues 16 to 31 serves as a PM-targeting signal, as amino acid changes in this region cause mislocalization of Gag to perinuclear compartments [2,16,17]. For example, a Gag mutant with changes in MA amino acids 29 and 31 (29KE/31KE) localizes at intracellular compartments positive for late endosome markers in HeLa and T cells [2]. This region may also play a role in Gag localization to the VCC in MDMs. A recent study observed that 29KE/31KE Gag localizes to VCC-like compartments in MDMs. However, these compartments appear to differ from VCCs containing WT Gag in that they are incapable of localizing to cell-cell junctions upon contact of infected MDMs with T cells [18]. Altogether, these studies demonstrate that the highly basic region of MA plays a critical role in Gag localization to the PM.

PI(4,5)P₂ as a potential determinant for Gag localization to the plasma membrane

Structural studies have established that the MA basic amino acids form a cluster on the surface of the MA globular domain and that such basic clusters are conserved among retroviruses [19], further supporting the importance of the MA basic region as a targeting signal. Then, what host factor serves as a counterpart for the basic region when Gag is targeted to the PM?

One candidate for such cellular binding partners of the MA basic region is a PM-specific acidic phospholipid, phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂]. PI(4,5)P₂ belongs to a family of lipids known as phosphoinositides that have a phosphorylated inositol ring in their head groups [20–24]. The number and positions of phosphate residues on this inositol ring differ between different phosphoinositides. Importantly, each of the phosphoinositides has a specific localization pattern in cells due to the different localizations of the enzymes generating or hydrolyzing each lipid species. It is known that a number of cellular proteins are targeted to the PM through interactions between the basic amino acid clusters (e.g., pleckstrin homology domain) and the head group of PI(4,5)P₂ [20–24]. In addition, in *in vitro* virus assembly experiments, IP5 and IP6 that are structurally related to the head group of PI(4,5)P₂ modulate Gag assembly to spherical particles [25]. Moreover, mathematical modeling suggested that

MA binds PI(4,5)P₂ preferentially over other acidic phospholipids [26]. Therefore, it is possible that cellular PI(4,5)P₂ is actively involved in HIV-1 assembly at the PM. To test this hypothesis directly, we examined impacts of cellular PI(4,5)P₂ perturbation on virus particle production [27]. To perturb cellular PI(4,5)P₂, we used two unrelated tools: 1) expression of constitutively active mutant of Arf6 (Arf6Q67L), which induces PI(4,5)P₂-enriched intracellular compartments, and 2) overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) that dephosphorylates D5 phosphate on the inositol ring of the PI(4,5)P₂ head group and effectively reduces the amount of cellular PI(4,5)P₂. When Arf6Q67L is expressed, Gag was relocalized to the newly formed PI(4,5)P₂-enriched compartments and as a result, extracellular virus release was reduced 2–3 fold [27]. Overexpression of 5ptaseIV in HeLa cells reduced virus particle production more severely [27]. Microscopy analyses showed that in 5ptaseIV-overexpressing cells, Gag fails to localize at the PM and instead concentrates to intracellular compartments including late endosomes (Fig. 2; data not shown) [27,28]. Additionally, a population of 5ptaseIV-overexpressing cells displayed only hazy cytosolic Gag localization indicating that general membrane binding of Gag is reduced in these cells [28]. Consistent with this interpretation, membrane flotation centrifugation experiments revealed that total Gag binding to membranes in cells is impaired by 5ptaseIV expression [28]. Together, these results suggest that PI(4,5)P₂ is essential for efficient Gag membrane binding as well as specific Gag localization to the PM. Similar impacts of PI(4,5)P₂ perturbation on virus particle production have been observed for Mason Pfizer monkey virus, murine leukemia virus, and HIV-2 [29–31], suggesting that the role played by PI(4,5)P₂ in virus particle production is conserved among retroviruses.

Gag binds PI(4,5)P₂ at membrane

What is the molecular mechanism by which PI(4,5)P₂ regulates Gag localization and membrane binding? It is most likely that Gag interaction with PI(4,5)P₂ recruits Gag to the PM due to the specific localization of this lipid to the PM. Consistent with this possibility, protein footprinting and solution NMR studies demonstrated that Gag derivatives (nonmyristylated Gag or myristylated MA domain) can interact with a water-soluble form of PI(4,5)P₂ that has truncated acyl chains [32,33]. Notably, the NMR study showed that the MA N-terminal myristate moiety becomes exposed upon PI(4,5)P₂ binding to MA [32]. The exposed myristate would be inserted into lipid bilayers, increasing the Gag binding to membrane containing PI(4,5)P₂. Therefore, this study suggests an interesting possibility that the PI(4,5)P₂ promotes Gag membrane binding both by serving as an anchor and by inducing myristate exposure. However, as both of these studies used nonnative PI(4,5)P₂ and Gag derivatives, it remained unclear whether WT Gag can interact with native, membrane-associated PI(4,5)P₂ as would be expected to occur in cells. To address this point, we developed an *in vitro* binding assay using myristylated full-length Gag and liposomes that contain native PI(4,5)P₂. In this assay, myristylated Gag synthesized by *in vitro* translation using rabbit reticulocyte lysates was incubated with liposomes containing various concentrations of PI(4,5)P₂ with native-length acyl chains, and liposome-bound Gag populations were separated from non-membrane-bound Gag by equilibrium flotation centrifugation. We observed that myristylation of Gag as well as PI(4,5)P₂ in liposomes are both required for efficient binding of Gag to liposomes [28](Fig. 2). These results indicate that WT Gag does bind membrane-associated PI(4,5)P₂ and that this interaction is essential for Gag membrane binding.

Determinants for the Gag-PI(4,5)P₂ interaction

The interaction between Gag and PI(4,5)P₂-containing liposomes is not simply due to electrostatic interactions. Increasing the content of a monovalent acidic phospholipid, phosphatidylserine, did not compensate for the lack of PI(4,5)P₂ for Gag binding to liposomes even though the overall negative charge of such liposomes was at or above the level of the

standard PI(4,5)P₂-containing liposomes [28]. Furthermore, substituting PI(4,5)P₂ with PI(3,5)P₂ significantly reduced the amount of Gag associated with liposomes [28]. Therefore, the interaction of Gag with PI(4,5)P₂ depends on not only the high density of the negative charge on the lipid head group, but also on the specific configuration of phosphate residues over the inositol ring.

To gain insights into determinants of Gag-PI(4,5)P₂ interaction, we further sought to identify Gag residues essential for this interaction. As mentioned above, the MA basic region, in particular Lys 29 and 31, plays an important role in proper localization of Gag to the PM [2, 17,34]. Therefore, we examined whether mutations at these MA basic residues abolish the PI(4,5)P₂-dependent Gag binding to liposomes. Indeed, substitutions of Lys 29 and 31 to acidic or neutral residues (29KE/31KE and 29KT/31KT, respectively) reduced Gag binding to PI(4,5)P₂-containing liposomes 2.5 fold, underlining the importance of the MA basic region for Gag interaction with PI(4,5)P₂ [28] (Fig. 2). A recent lipidomics study of HIV and MLV virions demonstrated that retroviral membranes are enriched with PI(4,5)P₂ compared to the PM of their producer cells [29]. This enrichment of PI(4,5)P₂ requires the MA globular domain that includes the highly basic region. Therefore, it is likely that MA interacts with PI(4,5)P₂ not only in *in vitro* systems but also in cells. Altogether, these studies indicate that the interaction of the MA highly basic region with PI(4,5)P₂ promotes proper localization of Gag to the PM and enhances membrane binding. Interestingly, substitutions of other basic residues in the MA basic region enhance Gag binding to liposomes not containing PI(4,5)P₂ and cause promiscuous localization of Gag in cells (Chukkapalli, Oh, and AO, submitted). Therefore, it is likely that the highly basic domain of MA regulates specific binding of Gag to PI(4,5)P₂-containing membranes by exerting dual and opposing functions. Further analyses of molecular interactions between PI(4,5)P₂ and the MA basic region is essential for gaining insights into therapeutic strategies that target the Gag-PI(4,5)P₂ interaction.

Gag localization after PM binding

After Gag is recruited to the PM through interaction with PI(4,5)P₂, does Gag stay there and form virus particle at the site of initial PM binding? In HeLa cells and other tissue culture cell lines, PM-associated Gag does not uniformly localize over the cell surface, but forms microscopically visible patches. Formation of these patches may involve clustering of lipid rafts or tetraspanin-enriched microdomains that have been shown to form virus assembly sites [35–44]. Notably, the NMR study suggests that when MA binds PI(4,5)P₂, the highly unsaturated 2' acyl chain of this lipid is sequestered inside the MA molecules [32] (Fig. 3). Sequestration of unsaturated acyl chains would likely increase the affinity of the Gag-PI(4,5)P₂ complex to lipid rafts. Therefore, it is possible that the MA-PI(4,5)P₂ interaction may regulate not only Gag binding to the PM but also partitioning of Gag to specific membrane microdomains.

It has been observed that heterogeneous Gag localization is even more pronounced in T cells, as Gag often concentrates to one pole of T cells [41,45,46]. It is possible that such accumulation of Gag and assembling virions to one end of cells may eventually facilitate virus spread through a cell-cell contact structure known as virological synapse [47–50]. The virological synapses, enriched with viral components as well as cell adhesion molecules and cytoskeletal proteins, are thought to facilitate transmission of HIV-1 and HTLV-1 due to higher frequency of virus assembly events at junction between virus-producing and target T cells [48,49,51–53]. However, the process leading to the formation of virological synapses largely remains to be elucidated. As the first step to address this point, we sought to determine Gag localization in mobile polarized T cells, since in lymphoid organs where contacts between infected and target T cells would likely occur more frequently, T cells are highly mobile and adopt a polarized morphology. By live cell microscopy of migrating primary T cells expressing Gag-GFP fusion

proteins, we observed that Gag localizes at the rear-end structure known as the uropod (Llewellyn and AO, manuscript in preparation). As this structure is enriched with multiple adhesion molecules [54], it is possible that contacts between T cells may be mediated by uropods decorated with abundant assembling or already assembled virus particles. Consistent with this hypothesis, we observed that Gag is highly concentrated at T-cell-T-cell contacts that are also positive for uropod markers (Llewellyn and AO, manuscript in preparation). These preliminary results support the possibility that in HIV-1-expressing T cells, the uropod may form a preassembled, virus-laden platform that serves as a precursor for the virological synapse. Consistent with this possibility, a recent live cell microscopy demonstrated that a preformed Gag-containing patch moves laterally toward the virological synapse [47]. Thus, localization of Gag to specific domains within the PM may facilitate virus spread to target cells. Our ongoing efforts are focused on understanding the mechanisms by which Gag accumulates to this specific area of the PM.

Concluding remarks

The PM is the major site of HIV-1 assembly. Recent data start to elucidate the molecular interactions promoting the proper localization of the viral proteins. Ensuring that virus assembly occurs at the PM and/or its particular domains is likely a critical process in the HIV-1 life cycle, as it affects extracellular virus release and transmission to the next target cells. Therefore, better understanding of the molecular mechanisms determining the sites of virus assembly will likely provide insights into new therapeutic strategies for suppressing HIV-1 propagation in infected individuals.

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Fig. 1.
A schematic representation of HIV-1 Gag domains. Structural domains and regions that function in steps of virus assembly are shown. N-terminal myristate moiety is shown (m-).

Fig. 2. PI(4,5)P₂ facilitates Gag localization at the PM and interaction with lipid bilayer. A. HeLa cells were transfected with an HIV-1 molecular clone expressing Gag-YFP fusion protein (Gag) and an expression plasmid encoding full-length myc-tagged 5ptaseIV (FL) or its inactive deletion mutant (Δ1). Note that in cells expressing full-length 5ptaseIV, Gag localizes at perinuclear compartments but not at the PM. B. [³⁵S]-labeled WT or 29KE/31KE Gag was synthesized by the *in vitro* transcription/translation system using rabbit reticulocyte lysates and incubated with liposomes containing indicated lipid components for 90 min. The mixture was subjected to membrane flotation centrifugation. Membrane (memb.)- and non-membrane (non-

memb.)-bound Gag was quantified by phosphorimager analysis. PC, phosphatidylcholine; PS, phosphatidylserine.

Fig. 3.

A proposed model for MA-PI(4,5)P₂ interaction and lipid raft association. A model proposed based on the NMR results [32] is depicted. Adapted from Reference [55].