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Molecular determinants that regulate plasma membrane association of HIV-1 Gag

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Abstract

HIV-1 assembly is a multi-step process that occurs at the plasma membrane. Targeting and binding of Gag to the plasma membrane are the first steps in this assembly process and are mediated by the matrix domain of Gag. This review highlights our current knowledge on viral and cellular determinants that affect specific interactions between Gag and the plasma membrane. We will discuss potential mechanisms by which the matrix domain might integrate three regulatory components, myristate, phosphatidylinositol-(4,5)-bisphosphate, and RNA to ensure that HIV-1 assembly occurs at the plasma membrane.

HIV-1 particle assembly is mediated by the viral structural protein Gag, which is synthesized as the precursor polypeptide Pr55^{Gag}. As defined by sites of the viral protease-dependent cleavage that occurs during or after virus release, this polypeptide has four major structural domains: matrix (MA), capsid (CA), nucleocapsid (NC) and p6^{1,2}. In addition, it also contains spacer peptides 1 and 2 (SP1 and 2). Each of these structural domains performs a crucial role during the assembly process. MA mediates the targeting of Gag to the site of assembly and facilitates membrane binding. The C-terminal domain of CA forms the Gag dimerization interface, whereas NC promotes higher order multimerization of Gag through binding to RNA, which is thought to serve as a scaffold. In addition, NC contains two zinc finger/knuckle structures that are required for specific encapsidation of viral genomic RNA. The late domain motifs in p6 recruit cellular protein complexes called endosomal-sorting complex required for transport (ESCRT) that aid in the release of virus particles from the cell membrane. During formation of infectious HIV-1 particles, several components need to be at the assembly site for their packaging into the virion, including Gag, GagPol, which contains all the viral enzymes, Env, viral RNA, and host factors that are required for virus infectivity, such as tRNA^{Lys3} and cyclophilin A.

HIV-1 assembly occurs at the plasma membrane (PM)³(Fig. 1) or, in the case of macrophages, in deep invaginations of the PM, which are connected to the cell surface via membrane-lined tubular conduits^{4–6}. Endosomes were also proposed previously to serve as native sites for assembly of HIV-1 particles as Gag was frequently observed in these compartments. However, most of the WT virus particles localized in these endosomal compartments are now thought to originate from endocytosis of particles already assembled

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at the PM⁷ (for reviews, see ^{8,9}). Consistent with the notion that endosomes do not constitute a native virus release pathway, redirecting Gag to endosomal compartments severely inhibits release of HIV-1 in HeLa, COS, and 293T cells perhaps because assembled particles are trapped in these compartments^{7,10–13}. It is therefore possible that the PM serves as the preferred site of HIV-1 assembly because it allows nascent particles to readily exit to the extracellular space. However, recent evidence suggests that at least in T cells, mutant Gag proteins targeted to the intracellular compartments can still be released as extracellular particles and are not necessarily trapped in these compartments¹⁴. Therefore, easy access to the extracellular space is unlikely to be the only reason that necessitates specific localization of HIV-1 Gag to the PM. While it remains to be determined what other advantages are associated with virus assembly at the PM, the molecular mechanisms by which Gag is specifically targeted to the PM are becoming clearer recently. In this review, we will summarize recent findings on the molecular determinants that regulate Gag-PM interaction, one of the earliest events during HIV-1 assembly, and discuss potential relationships between this event and other steps in virus assembly and post assembly processes.

Gag membrane binding is driven by bipartite signal in MA

MA is the membrane proximal domain of Pr55^{Gag}. The first 104 amino acids of the MA form a compact globular domain consisting of five major α -helices that are capped by a mixed three-stranded β -sheet^{15,16}. MA is myristoylated at the amino-terminus and facilitates membrane binding of Gag^{17,18}. In addition, a highly basic region (HBR) spanning residues 15–31 (Fig. 2A) is important for efficient membrane binding and proper targeting of Gag to the PM. These basic residues are clustered around the mixed β -sheet on the surface of MA and are thought to form interface with acidic phospholipids^{10–12,16,19–21}. Mutagenesis studies suggest that the region spanning residues 84–88 is also important for PM localization of Gag^{12,22}. However, this region is buried within MA and hence it is likely that the mutations in this region indirectly alter the HBR and affect Gag localization. Consistent with this hypothesis, a revertant of an MA mutant with a substitution in this region had a compensatory second site mutation in the MA HBR, suggesting that these regions are functionally linked²³. Besides having determinants for membrane binding and proper targeting, MA is also important for other processes in viral life cycle, such as envelope glycoprotein incorporation and post-entry processes [for review see ²⁴].

The N-terminal myristoyl moiety is sequestered in the globular domain of MA

Myristoylation is a process that occurs co-translationally, where a 14-carbon saturated fatty acid, myristic acid, is attached to the N-terminal glycine that is exposed after the first methionine is removed. The enzyme that catalyzes this reaction is the myristoyl-CoA: protein N-myristoyltransferase (NMT) [for detailed review see ²⁵]. Mutating the N-terminal glycine to alanine severely reduces membrane binding of Gag and inhibits virus particle release^{10,17,18,22,26}. In addition, mutating MA residues 2–5 (numbering of MA residues in this paper is based on the fact that glycine will be the first residue after the first methionine is removed) also reduces membrane binding and virus particle production, which is consistent with the idea that these residues are required for NMT recognition^{23,26}.

Differential membrane binding of the MA domain alone and of full-length Gag led to the hypothesis that a structural change in MA alters its myristate-mediated membrane binding^{27–30}. According to the original hypothesis of myristoyl switch, the myristate moiety is sequestered in the MA globular domain, and upon Gag multimerization, a structural change exposes the acyl chain facilitating efficient membrane binding. Consistent with this model, mutations of MA residues 6 and 7 were shown to inhibit myristate exposure^{26,31,32},

and certain other mutations or deletions in the MA globular domain were observed to enhance Gag membrane binding, presumably through inhibition of myristate sequestration^{21,26,27,29,32,33}. The presence of myristoyl switch was eventually demonstrated by NMR analysis³⁴. In this study, MA was shown to exist as an equilibrium mixture of myristate-sequestered monomer and myristate-exposed trimer. Notably, this study observed that the myristoyl moiety moves into or out of a hydrophobic cavity of MA in a manner coupled to MA trimerization without involving a drastic structural change of MA. Interestingly, C-terminal addition of the CA sequence, which contains a dimerization domain, shifted the equilibrium toward the myristate-exposed trimeric state by increasing the self-association of otherwise low-affinity molecules. These results suggest a model in which Gag multimerization stabilizes MA trimerization thereby facilitating myristate exposure and membrane binding.

Consistent with the multimerization-coupled myristoyl switch model, liposome or lipid monolayer binding studies showed that addition of the CA-derived dimerization domain to HIV-1 MA enhances its affinity for membranes^{35,36}. However, membrane binding of these Gag constructs was dependent on the presence of acidic phospholipids. Furthermore, similar observation was also obtained for RSV MA that lacks myristate³⁷. Therefore, these studies suggest that multimerization-dependent enhancement of membrane binding is explained at least partly by the increased avidity of the whole Gag complex to the membranes via clustering of multiple HBRs and myristate moieties on MA. In addition to the avidity, virus particle formation also could partly account for enhanced membrane binding of full-length Gag in cells, since Gag molecules within virus particles that remain on the cell surface are in effect irreversibly membrane-bound in fractionation experiments. Altogether, it is likely that the myristate exposure is not the sole reason for the difference between membrane binding of MA and that of full-length Gag²⁷⁻³⁰. Further in vivo and in vitro studies are required to sort out mechanisms contributing to the difference between membrane affinities of MA and full-length Gag.

Gag multimerization is not the sole trigger for exposure of the myristoyl moiety. As detailed later, the interaction of the HBR with a PM-specific acidic lipid, phosphatidylinositol-(4,5)-bispophosphate [PI(4,5)P₂] was also shown to trigger the myristate exposure for efficient membrane binding³⁸. Interestingly, non-myristoylated Gag that exists in a monomer-dimer equilibrium shifts to a monomer-trimer equilibrium when inositol hexakisphosphate (IP6) is added to the solution³⁹. As IP6 is structurally similar to the head group of PI(4,5)P₂, it is conceivable that PI(4,5)P₂ may also modulate trimerization of Gag, which is coupled to myristate exposure. In addition to PI(4,5)P₂, binding of calmodulin to MA and pH changes were shown to cause structural changes that modulate the status of myristate exposure^{40,41}. The role of calmodulin and pH changes in Gag membrane binding in cells has yet to be elucidated.

The MA HBR binds acidic lipids

Even though myristoylation is necessary, it is thought to be insufficient for efficient membrane binding of proteins^{42,43}. Myristoylation only provides reversible membrane binding, and a second signal is thought to be required for strong localization of the myristoylated protein to the membrane⁴³. This second signal can be a polybasic cluster, a second acylation such as palmitoylation, or a protein-protein interaction that increases avidity of the protein complex. In the case of HIV-1 Gag, the interaction of the HBR with acidic lipids can provide such second signal¹⁰.

The PM is one of the cellular membranes that are rich in anionic lipids such as phosphatidylserine (PS) and phosphoinositides that preferentially localize on the

cytoplasmic leaflet⁴⁴. This distinctive localization makes the cytoplasmic surface of the PM acidic. Proteins or protein domains with polybasic clusters on the surface, such as pleckstrin homology (PH) domains, myristoylated alanine-rich protein kinase C substrate (MARCKS), and k-ras, are targeted to this negatively charged surface where they interact with acidic lipids either in a lipid-head-group-specific manner or simply via electrostatic interactions^{45–47}. Mutations within the polybasic cluster of these proteins significantly alter their PM binding and localization^{48,49}. Similarly, mutations in the MA HBR mislocalize Gag to intracellular compartments and reduce membrane binding of Gag^{10–12,19–21}. Structural and mutagenesis studies suggest that the HBR is exposed on the surface of the MA domain and interacts with acidic lipids^{10,15,16,21,35,50}. Notably, however, Gag mutants lacking the MA globular domain except the N-terminal myristoylation signal can still bind membranes and release virus, suggesting that the HBR is important for efficient membrane binding only in the context of full-length MA^{33,51–54}.

Interaction between the MA HBR and phosphatidylinositol-(4,5)-bisphosphate is important for PM targeting and efficient membrane binding of Gag

As alluded to earlier, recent studies have shown that the basic residues in the HBR interact with the acidic phospholipid, PI(4,5)P₂^{21,38,55,56}. PI(4,5)P₂ belongs to a family of phospholipids called phosphoinositides. These lipids are derivatives of phosphatidylinositol and have a hydrophobic diacylglycerol backbone esterified to a polar inositol head group that can be phosphorylated at three of the five hydroxyl residues (Fig. 2B). Thus, seven different phosphatidylinositol phosphates (PIPs) are formed, which vary in the number and position of phosphates on the inositol ring⁵⁷. Various cellular proteins with basic residues on the molecule surface have different affinity and specificity to the head groups on phosphoinositides⁴⁶. Through the recruitment of these proteins in either a head-group-specific or simply a charge-dependent manner, PIPs control several important cellular processes including membrane fusion, budding, and actin dynamics^{58,59}. Notably, PIPs themselves are spatially and temporally regulated by the action of kinases and phosphatases that add or remove phosphate on the inositol ring, respectively. Among the PIPs, PI(4,5)P₂ is the most abundant and is specifically localized on the cytoplasmic leaflet of the PM.

The first clue for the role of PIPs in HIV-1 assembly came from an in vitro assembly study where both RNA and inositol pentakisphosphate (IP5) were required for Gag to form a proper virus-like structure⁶⁰. IP5 is an inositol derivative structurally related to the head group of PI(4,5)P₂. The requirement for PI(4,5)P₂ in HIV-1 assembly became evident from the study in which cellular PI(4,5)P₂ was depleted or mislocalized in HeLa cells, using either phosphatidylinositol polyphosphate 5 phosphatase type IV (5ptaseIV), which removes D-5 phosphate from the inositol head group⁶¹ or constitutively active Arf 6, which induces PI(4,5)P₂-enriched intracellular vesicles^{62,63}. Both methods of PI(4,5)P₂ perturbation mislocalized Gag to internal compartments and significantly reduced virus particle release¹³. Subsequently, several in vitro structural and biochemical studies showed that Gag interacts with PI(4,5)P₂ via basic residues in the HBR^{38,55,56,64}. Shkriabai, et al. examined the accessibility of lysines in non-myristoylated full-length Gag proteins after Gag was mixed with a water-soluble analogue of PI(4,5)P₂ that has short acyl chains and found two lysines in the MA HBR are masked from solvent by PI(4,5)P₂⁵⁶. Saad, et al. determined the structure of the complex between myristoylated MA and a water-soluble PI(4,5)P₂ analogue using NMR and identified binding of PI(4,5)P₂ to MA as a trigger for myristate exposure³⁸. Notably, in addition to interactions between the acidic head group of PI(4,5)P₂ and basic amino acids in the HBR, this study revealed the interaction between 2' acyl chain and a hydrophobic pocket that is normally occupied by the myristoyl moiety (Fig 3A). Consistent

with this finding, a study based on a surface plasmon resonance assay later showed that the presence of short acyl chains increased affinity of inositol phosphates to MA and Gag⁶⁵. While these studies utilized the water-soluble analogues of PI(4,5)P₂, studies using liposomes demonstrated that full-length myristoylated Gag or purified MA binds to membrane-associated PI(4,5)P₂ that contains native-length acyl chains^{55,64,66}. Gaining structural details of MA interaction with membrane-associated PI(4,5)P₂ will require the use of new techniques such as a recently developed NMR method that utilizes reverse micelle encapsulation⁶⁷. The notion that specific Gag-PI(4,5)P₂ interaction occurs in cells was supported by the detailed lipidomics study performed on HIV-1 particles. This study demonstrated that PI(4,5)P₂ is enriched in the HIV-1 membrane compared to the PM of virus-producing cells in an MA-dependent manner⁶⁸. Consistent with the role for PI(4,5)P₂ in efficient Gag-membrane binding observed in liposome-based experiments, ectopic expression of 5ptaseIV not only mislocalized Gag population to intracellular compartments but also increased non-membrane-bound Gag population in HeLa cells⁵⁵. In addition to HeLa cells, depletion of PI(4,5)P₂ in the natural host cells of HIV-1, T cells and macrophages, significantly reduced Gag-membrane association and virus release emphasizing the physiological relevance of the Gag-PI(4,5)P₂ interaction⁶⁹ (VC and AO unpublished data).

A recent study suggested that PI(4,5)P₂ has an additional function in HIV-1 assembly in which activation of inositol trisphosphate receptor by PI(4,5)P₂ hydrolysis and subsequent Ca²⁺ mobilization modulate endocytosis of Gag⁷⁰. Relationships between this indirect role of PI(4,5)P₂ and its role as a Gag binding partner remain to be elucidated.

Not only HIV-1, but other retroviruses including HIV-2, MLV and MPMV also require PI(4,5)P₂ for efficient virus particle production^{68,71–73}. However, not all retroviruses require PI(4,5)P₂. For example, although EIAV Gag that localizes both to the PM and intracellular compartments can bind PI(4,5)P₂⁷⁴, this Gag seems to depend on PI(3,5)P₂ rather than PI(4,5)P₂ for efficient virus release and membrane binding⁷⁵. Similarly, HTLV-1, which also localizes at the PM and intracellular compartments, is less sensitive to PI(4,5)P₂ depletion than HIV-1 and is able to bind membranes efficiently even in the absence of PI(4,5)P₂⁷⁶. Thus, different classes of retroviruses may have different requirements in terms of targeting to the site of assembly and virus particle production.

RNA bound to the MA HBR inhibits PI(4,5)P₂-independent membrane binding of Gag *in vitro*

In addition to binding acidic lipids, several *in vitro* studies have shown that the MA HBR can bind RNA^{56,77–83}. We hypothesize that RNA binding to MA could be a factor that necessitates the involvement of PI(4,5)P₂ for efficient membrane binding of Gag. We have recently shown that RNase treatment of Gag synthesized *in vitro* using rabbit reticulocyte lysate significantly increases binding of Gag to control liposomes that lack PI(4,5)P₂ but still contain another acidic lipid, phosphatidylserine (PS)²¹. These data suggest that RNA inhibits membrane binding of Gag, especially in the absence of PI(4,5)P₂. Consistent with these data, PI(4,5)P₂-containing liposomes, but not liposomes containing PS, out-competed oligonucleotides for binding to beads coated with purified MA domain⁶⁶. In addition to increased binding to acidic liposomes, RNase treatment of WT Gag also caused a modest increase in myristate-dependent binding to liposomes containing only a neutral lipid, PC²¹. Thus, at least in *in vitro* experiments, RNA is likely to act as a negative regulator of non-specific membrane binding by blocking both electrostatic interactions of Gag with acidic lipids and to a lesser extent hydrophobic interactions determined by myristate exposure. Unlike PI(4,5)P₂, however, there is little data that provide insights into the role of MA-bound RNA in Gag- membrane binding in cells. Ongoing studies in our laboratory suggest

that not only Gag proteins synthesized in reticulocyte lysates but also Gag derived from cells are susceptible to similar RNA-mediated inhibition of membrane binding (VC and AO, unpublished data). Currently, however, the identity of the RNA involved in this inhibition and the molecular determinants for the MA-RNA interaction that causes this inhibition remain unknown. What would be the implication of such MA-RNA interaction in the viral life cycle? MA-viral RNA interaction has been shown to be important for genomic RNA dimerization, encapsidation, or nucleocytoplasmic transport of Gag in other retroviruses^{84–87}, but there is no report indicating a similar role for this interaction in HIV-1 life cycle. Nevertheless, there are several recent studies functionally linking nuclear export of HIV-1 gRNA and MA-mediated membrane binding. It has been known that HIV-1 assembly is blocked in murine cells^{88–91}. However, altering the RNA export pathway or introducing MA mutations that enhance Gag membrane binding^{31–33,92} was sufficient for alleviating the block in HIV-1 assembly in murine cells⁹³. Similar MA-dependent inhibition of assembly was also observed in human cells, when the classical Rev response element (RRE) was replaced with Hepatitis B virus post-transcriptional regulatory element (PRE)⁹⁴. Thus, these studies suggest that the membrane binding of Gag mediated by MA is functionally linked to the RNA export pathway used by HIV-1. The nature of the link between RNA export and membrane binding has yet to be elucidated.

MA-PI(4,5)P₂ binding may be important for coordinating Gag membrane binding with other assembly and post-assembly events

As mentioned earlier, both myristate and HBR are necessary for efficient membrane binding of Gag. Either one alone is not sufficient in the context of full-length Gag in cells, as supported by observations that both non-myristoylated Gag and Gag derivatives with substitutions of all basic residues in the HBR do not bind membranes efficiently^{10,17,19,21,26}. However, in contrast to amino acid substitutions in the HBR, deletion of the entire MA globular head except for the myristoylation signal enhances Gag membrane binding^{33,51–54}. Treating non-myristoylated Gag with RNase also rescues binding of this otherwise defective Gag to membranes that contain PS, an abundant acidic phospholipid in the inner leaflet of cell membranes²¹. These observations suggest that membrane binding is inhibited by the globular-head-dependent sequestration of myristate and potentially by RNA bound to the HBR. In this context, it is intriguing that PI(4,5)P₂ appears to be capable of reversing both modes of inhibition. Unlike PS, PI(4,5)P₂ is able to bind MA HBR even in the presence of RNA^{21,66}, perhaps because it binds to a site in MA not blocked by RNA or has a higher affinity for MA than the RNA. NMR studies on MA-PI(4,5)P₂ interaction suggest that binding of Gag to PI(4,5)P₂ causes a structural change that can expose myristate³⁸. Thus, both targeting of Gag to PI(4,5)P₂-enriched membranes, i.e. PM, and efficient membrane binding are tightly linked.

NMR data further suggest that once the myristoyl moiety is exposed, the hydrophobic pocket of Gag is then occupied by the 2' acyl chain of PI(4,5)P₂ that is usually unsaturated³⁸. This would leave two saturated acyl chains that mediate MA-membrane interaction; the remaining saturated 1' acyl chain of PI(4,5)P₂ and the N-terminal myristoyl moiety of Gag (Fig. 3A). The intriguing implication of this association is that it could enhance the affinity of Gag for PM microdomains known as lipid rafts, where saturated but not unsaturated acyl chains are preferred. Consistent with this model, replacing N-terminal myristate with an unsaturated analog significantly reduces Gag in detergent resistant membrane fractions that are thought to originate from lipid rafts⁹⁵. Lipid rafts have been previously shown to play a crucial role in virus assembly and infectivity of progeny virus particles^{95–99} (for review see^{100,101}). Therefore, interaction with PI(4,5)P₂ not only promotes specific Gag localization to the PM and stable membrane binding, but may also

confer additional advantage to HIV-1 by associating assembly events to lipid rafts. The latter role for PI(4,5)P₂ has not been tested experimentally.

As mentioned earlier, *in vitro* studies indicate that RNA bound to MA HBR can block interactions with PS but not with PI(4,5)P₂^{21,66}. Therefore, it is possible that the requirement for PI(4,5)P₂ could be a mere consequence of RNA-mediated inhibition of MA interaction with other acidic lipids. Conversely, RNA may be recruited to ensure specificity of the MA HBR for PI(4,5)P₂ and membranes containing this lipid (i.e., the PM). Indeed, HTLV-1 Gag membrane binding that is less dependent on PI(4,5)P₂ is also not inhibited by RNA, suggesting a correlation between RNA binding and PI(4,5)P₂-dependent membrane interaction⁷⁶. Interestingly, a neutron scattering study showed that in solution, monomeric Gag adapts a conformation in which Gag is folded over, and MA and NC are in a close proximity¹⁰². When bound to either acidic membrane or oligonucleotides, Gag is still in a compact shape. However, when both acidic membrane and oligonucleotides are present, Gag forms the extended rod-like structure similar to what is observed in immature virions¹⁰³ (Fig. 3B). An intriguing model proposed in these studies is that when bound to either the PM or RNA, Gag can do so via both basic regions in MA and NC simultaneously. When both the PM and RNA are present, however, MA-PM and NC-RNA interactions may occur in parallel. Thus, it is conceivable that once RNA-bound Gag reaches the PM, a high affinity of the MA HBR to PI(4,5)P₂ may help strip off RNA from MA, but not NC (Fig. 3B). Consistent with this possibility, *in vitro* RNA chaperone activity (annealing of tRNA to the primer-binding site of viral RNA) of NC in the context of Gag increased several fold when IP6 was added to Gag or when MA was deleted¹⁰⁴. These data suggest that RNA binding of MA hinders NC activities in the absence of inositol phosphates. Based on the above data, we speculate that RNA-mediated regulation of membrane binding may be a way of coordinating several major assembly steps for HIV-1. Once the MA HBR is bound to PI(4,5)P₂ and releases RNA, NC may become capable of freely performing its functions, such as tRNA annealing and RNA-mediated Gag multimerization. However, it is important to note that this model assumes that the same RNA binds both MA and NC, an assumption that has to be tested experimentally.

Interestingly, NC-mediated multimerization has been shown to be important for localization of Gag to the rear-end protrusions known as uropods in polarized T cells¹⁰⁵. Therefore, binding of PI(4,5)P₂ to MA might represent the transition from the MA-driven PM-targeting phase to the NC-dependent uropod localization phase. Notably, these Gag-containing uropods form frequent contacts with other T cells and participate in virological synapses^{105,106} at which cell-to-cell virus transmission is thought to take place efficiently^{107–110} (for review see^{111–115}). Therefore, this polarized localization of Gag on the PM likely facilitates efficient cell-to-cell transfer of the virus at the contact sites, which might represent another advantage associated with targeting of Gag to the PM. Further studies are required to better understand the connection between the MA-mediated regulation of targeting and membrane binding of Gag and the NC-mediated Gag multimerization step that drive Gag to specific sites within the plane of the PM.

Conclusions

Binding of Gag to the PM is the essential early step in HIV-1 assembly. These processes are likely modulated by MA in three different but coordinated ways: positively by interacting with PI(4,5)P₂, negatively by suppressing myristate-mediated hydrophobic interaction, and negatively by interacting with RNA. With the present knowledge on Gag-membrane binding, several types of drugs can be developed for inhibiting this process during HIV-1 assembly. Blocking N-terminal myristoylation of Gag or supplementing cells with unsaturated analogues of myristate has already been shown to significantly reduce infectious

virus particle production^{95,116–119}. Similarly, as Gag- PI(4,5)P₂ interaction plays an essential role in virus assembly, small molecule inhibitors that inhibit this interaction can be developed. Lately, RNA aptamers have been emerging as a new class of therapeutics for several different diseases¹²⁰. With the recent evidence for the role of RNA in the inhibition of membrane binding of Gag, RNA aptamers that bind MA with higher affinity than PI(4,5)P₂ might prove useful as inhibitors of HIV assembly (for example, see reference⁸³).

Mechanisms regulating both MA-PI(4,5)P₂ and MA-RNA interactions, as well as their potential roles in coordinating Gag membrane binding and other steps in assembly and post-assembly processes remain to be elucidated. In addition, several events that precede Gag-membrane binding continue to pose outstanding questions: Where is Gag synthesized? How does Gag traffic to the PM at which PI(4,5)P₂ stabilizes its membrane binding? When and where does Gag encounter the RNA that inhibits MA-dependent membrane binding? How and why are RNA-export pathway and membrane binding linked? Addressing these questions will provide new strategies to inhibit HIV-1 replication at early stages of virus assembly.

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Abbreviations

HIV-1	HIV type 1
MA	matrix
HBR	highly basic region
PI(4,5)P₂	phosphatidylinositol-(4,5)-bisphosphate
CA	capsid
NC	nucleocapsid
PM	plasma membrane
WT	wildtype

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Figure 1. HIV-1 assembly on the plasma membrane

HeLa cells were transfected with HIV-1 molecular clones that encode Gag tagged with Venus fluorescent protein (A) or WT Gag (B and C), fixed, and analyzed by epifluorescence microscopy (A) or electron microscopy (B and C), respectively. Higher magnification image of the area corresponding to the black rectangle in B is shown in C. Note that GagVenus puncta and virus particle formation are seen on the plasma membrane.

Figure 2. The MA domain and PI(4,5)P₂

A) Schematic representation of the bipartite membrane binding signal within the MA domain (yellow) of Gag. The N-terminal myristoyl moiety (green) and the highly basic region (blue), both of which are important for membrane binding of Gag, are shown. The amino acid sequence of the highly basic region corresponds to that of HIV-1_{NL4-3} MA. B) Structure of PI(4,5)P₂. PI(4,5)P₂ has a polar inositol head group, which is phosphorylated at D-4 and D-5 on the inositol ring. The acyl chains of PI(4,5)P₂ found commonly in cells are saturated stearic acid at position 1' and unsaturated arachidonic acid at position 2'.

Figure 3. Regulation of Gag-plasma membrane binding via MA-PI(4,5)P₂ and MA-RNA interactions

A) A model of MA-PI(4,5)P₂-membrane interaction reprinted from reference (38) is shown (Copyright, Michael F. Summers, used with permission). Upon PI(4,5)P₂ (yellow with red phosphates) binding to the MA HBR (lysine and arginine side chains shown in blue), the myristate (green) is exposed, and instead 2' acyl chain of PI(4,5)P₂ occupies the hydrophobic pocket of MA. The exposed myristate moieties and 1' acyl chains of PI(4,5)P₂ projected from MA are expected to synergistically promote membrane binding. B) A possible mechanism by which PI(4,5)P₂ and RNA regulate Gag binding to the plasma membrane is shown. In this schematic representation, both sequestration of the N-terminal myristoyl moiety and binding of RNA to the HBR inhibit non-specific membrane binding of MA via hydrophobic and electrostatic interactions, respectively. Once Gag reaches the plasma membrane, PI(4,5)P₂ binds to the MA HBR thereby serving as a membrane anchor for Gag even when RNA is bound to the HBR. In addition, PI(4,5)P₂ binding to MA exposes myristate, further enhancing membrane binding. Altogether, these positive and negative regulatory mechanisms ensure the plasma membrane specificity of Gag. Upon PI(4,5)P₂ binding, sequestration of unsaturated 2' acyl chain of PI(4,5)P₂ into a hydrophobic

pocket vacated by the myristoyl moiety may result in Gag association with membrane via two saturated acyl chains as shown in panel A. This is likely to increase propensity of Gag to partition into lipid rafts. In addition, MA-PI(4,5)P₂ interaction might induce dissociation of RNA from MA, which in turn may facilitate NC-mediated functions, such as higher-order multimerization. Therefore, it is possible that MA-PI(4,5)P₂ interaction coordinates Gag membrane binding with other assembly processes.