Opposing mechanisms involving RNA and lipids regulate HIV-1 Gag membrane binding through the highly basic region of the matrix domain

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Membrane binding of Gag, a crucial step in HIV-1 assembly, is facilitated by bipartite signals within the matrix (MA) domain: N-terminal myristoyl moiety and the highly basic region (HBR). We and others have shown that Gag interacts with a plasma-membrane-specific acidic phospholipid, phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂], via the HBR, and that this interaction is important for efficient membrane binding and plasma membrane targeting of Gag. Generally, in protein-PI(4,5)P₂ interactions, basic residues promote the interaction as docking sites for the acidic headgroup of the lipid. In this study, toward better understanding of the Gag-PI(4,5)P₂ interaction, we sought to determine the roles played by all of the basic residues in the HBR. We identified three basic residues promoting PI(4,5)P₂-dependent Gag-membrane binding. Unexpectedly, two other HBR residues, Lys25 and Lys26, suppress membrane binding in the absence of PI(4,5)P₂ and prevent promiscuous intracellular localization of Gag. This inhibition of nonspecific membrane binding is likely through suppression of myristate-dependent hydrophobic interaction because mutating Lys25 and Lys26 enhances binding of Gag with neutral-charged liposomes. These residues were reported to bind RNA. Importantly, we found that RNA also negatively regulates Gag membrane binding. In the absence but not presence of PI(4,5)P₂, RNA bound to MA HBR abolishes Gag-liposome binding. Altogether, these data indicate that the HBR is unique among basic phosphoinositide-binding domains, because it integrates three regulatory components, PI(4,5)P₂, myristate, and RNA, to ensure plasma membrane specificity for particle assembly.

basic residues | PI(4,5)P₂ | phosphoinositide | plasma membrane | retrovirus assembly

HIV-1 assembly is a multistep process mediated primarily by the viral structural protein, Gag. This protein, synthesized as a polyprotein precursor, Pr55^{Gag}, contains four major domains: matrix (MA), capsid (CA), nucleocapsid (NC), and p6. CA and NC contain determinants for Gag multimerization, whereas p6 facilitates release of virus particles (1, 2). In most cell types, Gag assembles on the cytoplasmic leaflet of the plasma membrane (PM). MA is required for this PM-specific targeting as well as the lipid bilayer binding of Gag.

MA has bipartite entities that facilitate membrane binding of Gag: (i) the N-terminal myristoyl moiety and (ii) a highly basic region (HBR) comprising of residues 17–31 (3–5). The N-terminal myristate is normally sequestered within the globular head domain of MA (6), which also includes the HBR. A structural change, either through Gag multimerization or phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] binding to MA, is required to expose the myristate and enhance membrane binding (6, 7). This mechanism is known as the myristoyl switch (8, 9). Structural and biochemical studies suggest that the HBR of HIV-1 MA and analogous regions of other retroviral MA are apposed to face the cytoplasmic leaflet of membranes and facilitate membrane binding by interacting with acidic lipids (4, 10–14). In addition, MA, especially the HBR, has been shown to interact with RNA (15–19) although the role of this interaction in virus assembly is less well

understood. The HBR is also implicated in other steps of virus replication such as the postentry process (for review, see ref. 20).

Many cellular proteins that interact peripherally with the cytoplasmic leaflet of membranes have basic amino acid-rich domains (21). The subcellular localization of these proteins generally depends on the specific binding of their basic domains with acidic lipids of target membranes. For example, the pleckstrin homology domain of phospholipase C δ 1 (PH_{PLC δ 1}) has basic residues located on the surface of a binding pocket for PI(4,5)P₂, a PM-specific acidic lipid. These basic residues specifically interact with the phosphates on the inositol headgroup of PI(4,5)P₂ (22). This interaction allows PH_{PLC δ 1} to localize to the PM. Similarly, basic residues of other phosphoinositide-binding domains play crucial roles in specific interactions with target membranes.

Previously, we showed that depleting cellular PI(4,5)P₂ reduces virus release of HIV-1 significantly and relocalizes Gag from the PM to cytosol or intracellular compartments (23, 24). Not only HIV-1, but other retroviruses such as HIV-2, murine leukemia virus, and Mason-Pfizer monkey virus also require PI(4,5)P₂ or other phosphoinositides for efficient virus release (25–28). Several studies using various *in vitro* techniques, including NMR, protein foot printing, liposome binding, and monolayer membrane binding, suggest that MA interacts specifically with PI(4,5)P₂ (7, 24, 29, 30). A recent lipid analysis study demonstrated that PI(4,5)P₂ is enriched in the HIV envelope in an MA-dependent manner, confirming the MA-PI(4,5)P₂ interaction *in vivo* (26). These studies suggest that MA basic residues, in particular those in the HBR, promote PI(4,5)P₂ interaction and membrane binding, as observed for basic residues in other phosphoinositide-binding domains. However, the contribution of each basic residue in the HBR to the interaction between full-length Gag and PI(4,5)P₂-containing membrane remains to be examined.

Previous reports showed that mutating lysines 29 and 31 to threonines (29KT/31KT) within the HBR relocalizes Gag from the PM to intracellular compartments, as observed for wild-type (WT) Gag in PI(4,5)P₂-depleted cells (31). Consistent with the altered localization, the *in vitro* liposome binding assay showed that 29KT/31KT Gag binds PI(4,5)P₂ less efficiently compared to WT Gag (24). However, the Gag-PI(4,5)P₂ interaction was not completely abolished, suggesting a role for other basic amino acids in this interaction.

In this study, toward better understanding of the Gag-PI(4,5)P₂ interaction, we sought to identify all of the basic residues in the HBR important for this interaction. We confirmed that the HBR as a whole is indeed essential for PI(4,5)P₂ interaction and efficient

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Fig. 4. MA-bound RNA suppresses membrane binding of Gag by inhibiting interactions between basic residues and acidic lipids and by reducing the myristate-dependent interaction. (A) Liposome binding of WT and delNC Gag was analyzed as in Fig. 2 by using PC+PS liposomes. Data from three independent experiments are shown as means $\pm$ SD. **, $P < 0.01$. There is no statistically significant difference in membrane binding of RNase-treated Gag between WT and delNC. (B and C) Liposome binding of WT and 1GA Gag was analyzed as in Fig. 2 by using liposomes containing either PC+PS or PC+PS+PI(4,5)P₂ (B) or only PC (C). Data from three to five independent experiments are shown as means $\pm$ SD. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. There is a statistically significant difference (*) in binding to PC+PS liposomes but not PI(4,5)P₂-containing liposomes between WT and 1GA Gag treated with RNase.

Gag. These results are consistent with the possibility that RNA also inhibits membrane binding partially by sequestration of myristate. Indeed, WT Gag did show a modest increase in PC-

liposome binding upon RNase treatment, whereas 1GA Gag showed no increase (Fig. 4C). These data suggest that removal of RNA has an impact on the myristoyl switch as well. Altogether, these results indicate that RNA inhibits membrane binding both by preventing the electrostatic interaction between basic residues and acidic lipids and by suppressing the myristate-dependent hydrophobic interaction.

Discussion

Phosphoinositide-binding domains found in cellular proteins contain basic residues that are essential for interaction with acidic headgroups of phosphoinositides (21). Similarly, the current study showed that some basic residues within the HBR (Lys17, Lys29, and Lys31) are important for efficient Gag membrane binding mediated by PI(4,5)P₂ (Fig. 1A). However, our study also revealed that the HBR contains basic residues (Lys25 and Lys26) that suppress membrane binding of Gag (Fig. 1A). This negative regulation of membrane binding by Lys25 and Lys26 likely occurs via myristate sequestration (Fig. 3). Consistent with the increased PI(4,5)P₂-independent liposome binding, 25KT/26KT Gag localizes promiscuously in cells and releases virus particles more efficiently than WT Gag (Fig. 1B and C), a phenotype reminiscent of Gag derivatives with MA globular head deletions (35).

This work also identified RNA as a negative regulator of Gag membrane binding. Several *in vitro* studies including this study (Fig. S3) showed that HIV-1 MA interacts with RNA via its HBR (17–19). Therefore, competition between RNA and acidic lipids for binding to the HBR at least partly accounts for the RNA-mediated suppression of Gag membrane binding. Consistent with this hypothesis, in other *in vitro* experiments performed in the absence of RNA, binding of MA to PS has been

Fig. 5. A model for the regulation of Gag-membrane binding specificity. PI(4,5)P₂-dependent and -independent membrane binding of WT, 25KT/26KT, and RNase-treated Gag are shown. See Discussion for explanation.

readily observed (36–38), whereas in our system, Gag binding to the PC+PS liposomes was negligible without RNase treatment. Importantly, in contrast to PC+PS liposomes, a substantial amount of Gag binds to PC+PS+PI(4,5) P_2 liposomes even without RNase treatment (Figs. 2 and 4). Consistent with these data, while this manuscript was in revision, Eric Barklis's group reported that PI(4,5) P_2 -containing liposomes out-compete oligonucleotides for binding to beads coated with purified myristylated MA domain (39). Thus, it is likely that PI(4,5) P_2 , but not other acidic lipids such as PS, can bind the HBR in the presence of RNA. In addition, exposure of myristate may also be more dependent on PI(4,5) P_2 binding in the presence of RNA, because RNA binding to MA HBR also reduces myristate-dependent hydrophobic interaction (Fig. 4). In any case, our data suggest that RNA binding to MA represents an important regulatory mechanism in Gag membrane binding.

It remains to be determined whether RNA bound to the HBR inhibits unregulated membrane binding in cells. In our *in vitro* system, the RNA concentration is estimated to be lower than that in the cytoplasm of HeLa cells. Nonetheless, the inhibition of membrane binding by RNA does not require NC, the major RNA binding domain (Fig. 4A). However, in cells, it is likely that the viral genomic RNA bound to NC also binds MA because of their close proximity. This model is also consistent with a recently proposed folded-over conformation of Gag (40, 41) (not depicted in Fig. 5). Notably, two recent reports suggest that RNA export from the nucleus plays a key role in MA-mediated membrane binding of Gag (42, 43). These authors observed that HIV Gag assembly defects linked to specific Gag mRNA export pathways could be rescued by increasing membrane binding ability of Gag, either by MA mutations or replacing MA with heterologous membrane binding domains (42, 43). In light of the results presented here, it is possible that nuclear export pathways used by viral RNAs affect MA–RNA interactions that, in turn, influence Gag membrane binding. This hypothesis is also in agreement with the observed autoinhibition of Gag membrane binding by HIV-1 MA in murine cells (44, 45).

It has been long known that retroviral MA interacts with RNA (15–19, 46–50). Although genomic RNA encapsidation does not require MA in the case of HIV-1 (51), a variety of functions have been suggested for the retroviral MA–RNA interaction in the later half of the virus life cycle. These functions include (i) regulation of viral mRNA translation (18, 52), (ii) encapsidation of genomic viral RNA (53), (iii) dimerization of encapsidated RNA (54, 55), and (iv) enhancement of Gag assembly (56, 57). Thus, it is tempting to speculate that binding of the HBR to both PI(4,5) P_2 and RNA may play a key role in coordinating the four-way interactions between the PM, viral RNA, MA, and NC during the late phase of the retrovirus life cycle. For example, once Gag reaches the PM, the viral RNA bound to MA might get displaced by the HBR–PI(4,5) P_2 interaction. This displaced RNA may then act as a scaffold for NC-dependent Gag multimerization and facilitate efficient particle assembly at the PM. Such a process might also require cellular proteins reported to play a role in Gag multimerization or RNA packaging, including ATP-binding cassette protein in the E subfamily (ABCE1) (58, 59) and Staufen-1 (60). Clearly, further studies are necessary for addressing interrelationships between events during the late phase.

In summary, our data indicate that the HBR within MA modulates membrane binding in three different ways: positively by interaction with PI(4,5) P_2 , negatively by suppression of myristate-

dependent hydrophobic interaction, and negatively by interaction with RNA. RNA binding to MA HBR not only interferes with interactions between MA basic amino acids and acidic lipids, but also appears to reduce myristate exposure. Based on this and previous studies, we put forth the following model (Fig. 5). Both RNA binding and suppression of myristate exposure mediated by the HBR prevent Gag from premature or nonspecific membrane binding. Once Gag reaches PI(4,5) P_2 -containing membranes such as the PM, PI(4,5) P_2 binds the HBR, which then triggers myristate exposure and stabilizes binding to the lipid bilayer. However, if myristate sequestration is blocked (e.g., by the 25KT/26KT mutations) or if RNA detaches from the HBR (e.g., by RNase treatment), Gag binds membrane in a nonspecific, PI(4,5) P_2 -independent manner (Fig. 5). Cooperation of interactions with multiple membrane factors, which enhances the robustness and specificity of membrane binding, is prevalent in phosphoinositide-binding domains (21). However, MA HBR is unique among these domains for integrating opposing regulatory mechanisms to ensure specificity for the target membrane that contains PI(4,5) P_2 .

Materials and Methods

Cells and Plasmids. HeLa cells were cultured and maintained in DMEM supplemented with 5% FBS. HIV-1 molecular clone pNL4-3 (61), its derivative encoding YFP-tagged Gag (pNL4-3/GagVenus) and a Gag expression vector for *in vitro* translation reaction, pGEMNLNR, were described (24). pNL4-3/1GA, pNL4-3/25KT/26KT, pNL4-3/17KT/19RL, pNL4-3/29KT/31KT, pNL4-3/19RL, and pNL4-3/21RL were kind gifts from E. Freed (HIV Drug Resistance Program, National Cancer Institute, Frederick, MD) (33, 34). Other MA changes were introduced into pNL4-3 by PCR mutagenesis. MA changes in pNL4-3 derivatives were also introduced into pGEMNLNR and pNL4-3/Gag-Venus by using standard molecular biology techniques. pGEMNLNR/delNC was constructed by replacing BssHII to PflMI fragment (nt 711–5297) with that of pNL4-3/delNC (a kind gift from D. Ott) (AIDS Vaccine Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD) (62).

Liposome Binding Assay. Liposome binding assay was performed as described (24). Briefly, radiolabeled Gag is synthesized by using *in vitro* transcription and translation coupled reaction mix containing rabbit reticulocyte lysates (Promega). Liposomes containing corresponding lipids were added after 30 min at 30°C and incubated further for 90 min. Then, the liposome-bound and non-liposome-bound Gag were separated by equilibrium flotation centrifugation.

For RNase treatment experiments, the above protocol was modified as following. Gag synthesis was performed at 30°C for 90 min. Subsequently, 1 μ L of RNase, which is a mixture of pyrimidine-specific endoribonucleases from bovine pancreas (Cat. No.: 11119915001, Roche Applied Science), was added to 25 μ L of the reaction mix and incubated for 20 min at 37°C. Liposomes were then added and incubated for 15 min at 30°C before equilibrium flotation centrifugation was performed.

Virus Release Assay, Microscopy, and Statistical Analysis. Virus release assay was performed as described (23). Microscopy and statistical analysis were performed as described (24).

Coimmunoprecipitation of Gag and RNA. RNA binding to Gag synthesized in *in vitro* transcription and translation coupled reactions was analyzed as described in *SI Materials and Methods*.

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