Interaction between the Human Immunodeficiency Virus Type 1 Gag Matrix Domain and Phosphatidylinositol-(4,5)-Bisphosphate Is Essential for Efficient Gag Membrane Binding[▽]

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Human immunodeficiency virus type 1 (HIV-1) particle assembly mediated by the viral structural protein Gag occurs predominantly on the plasma membrane (PM). Although it is known that the matrix (MA) domain of Gag plays a major role in PM localization, molecular mechanisms that determine the location of assembly remain to be elucidated. We observed previously that overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) that depletes PM phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] impairs virus particle production and redirects processed Gag to intracellular compartments. In this study, we examined the impact of PI(4,5)P₂ depletion on the subcellular localization of the entire Gag population using Gag-fluorescent protein chimeras. Upon 5ptaseIV overexpression, in addition to perinuclear localization, Gag also showed a hazy cytosolic signal, suggesting that PI(4,5)P₂ depletion impairs Gag membrane binding. Indeed, Gag was less membrane bound in PI(4,5)P₂-depleted cells, as assessed by biochemical analysis. These observations are consistent with the hypothesis that Gag interacts with PI(4,5)P₂. To examine a putative Gag interaction with PI(4,5)P₂, we developed an in vitro binding assay using full-length myristoylated Gag and liposome-associated PI(4,5)P₂. Using this assay, we observed that PI(4,5)P₂ significantly enhances liposome binding of wild-type Gag. In contrast, a Gag derivative lacking MA did not require PI(4,5)P₂ for efficient liposome binding. To analyze the involvement of MA in PI(4,5)P₂ binding further, we examined MA basic amino acid substitution mutants. These mutants, previously shown to localize in perinuclear compartments, bound PI(4,5)P₂-containing liposomes weakly. Altogether, these results indicate that HIV-1 Gag binds PI(4,5)P₂ on the membrane and that the MA basic domain mediates this interaction.

Retroviral particle production is a complex multistep process mediated by a viral structural protein, Gag. Human immunodeficiency virus type 1 (HIV-1) Gag is synthesized as a precursor polyprotein, Pr55^{Gag}. This precursor polyprotein consists of four major domains which, upon virus release, are cleaved by the viral protease (PR) to generate the mature Gag proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6. In addition, Pr55^{Gag} contains two spacer peptides, SP1 and SP2 (2, 21, 83). MA, which constitutes the N-terminal domain of Pr55^{Gag}, is important for Gag membrane binding and targeting of Gag to the plasma membrane (PM). CA and NC promote Gag multimerization, and p6 is essential for the release of virus particles (2, 21, 83).

Membrane binding of HIV-1 Gag is mediated by two signals in MA: the N-terminal myristic acid and the conserved basic region between MA amino acids 17 and 31 (8, 26, 93). The myristate moiety is considered to be regulated by a mechanism termed a myristoyl switch (30, 57, 65, 72, 74, 75, 80, 84, 94). In this model, the N-terminal myristate is normally sequestered in the MA globular domain, but a structural change exposes myristate and enhances Gag membrane binding. The patch of conserved basic residues likely contributes to membrane bind-

ing by interacting with acidic phospholipids in the inner leaflet of the PM (32, 69, 93). In addition, the MA basic domain is involved in specific localization of Gag to the PM. In HeLa and T cells, mutations in the basic domain of MA shift the Gag localization from PM to intracellular vesicles containing late endosomal marker proteins (24, 31, 58, 61, 91). In apparent contrast to HeLa and T cells, in macrophages, even when wild-type (WT) Gag is expressed, the virus particles localize primarily at compartments positive for late endosomal markers (52, 58, 66, 70). However, these compartments, which first appeared to be internal, were later shown to be invaginations of the PM (17, 88). Moreover, even in macrophages, a population of Gag was clearly observed at the cell surface, especially at early time points (37). This specific localization of Gag to the PM or PM-related compartments suggests that host cellular factors play a role in determining the site of virus assembly.

Many cellular proteins that bind the PM have membrane-targeting sequences, such as pleckstrin homology (PH) domains. These domains are known to have clusters of basic amino acids that interact with negatively charged lipids collectively known as phosphoinositides (18, 35, 41). Different phosphoinositides localize in different subcellular membranes, thereby influencing the location of proteins to which they bind (79, 89). Phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂] is concentrated primarily on the cytoplasmic leaflet of the PM, which is also the site where Gag assembles in most cell types. As mentioned above, Gag also contains a patch of basic amino acids that are important for PM localization. Furthermore, IP5

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and IP6, which are structurally similar to PI(4,5)P₂, were shown to interact with Gag in in vitro Gag assembly studies (10, 16). Altogether, this information led to the hypothesis that PI(4,5)P₂ plays a role in PM localization of Gag through interaction with the MA basic amino acids.

Previously, we showed that perturbing PM PI(4,5)P₂ in HeLa cells markedly reduces virus production. This was demonstrated using two different approaches: overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) and a constitutively active ADP-ribosylation factor 6 (Arf6) mutant, Arf6/Q67L (55). Overexpression of 5ptaseIV reduces cellular PI(4,5)P₂ levels by hydrolyzing the phosphate group at the D5 position of the inositol ring (38), whereas expression of Arf6/Q67L induces PI(4,5)P₂-enriched vesicles within the cytoplasm (3, 7). Altering PI(4,5)P₂ levels by 5ptaseIV overexpression drastically reduced virus release efficiency compared to cells expressing a 5ptaseIV Δ 1 mutant that lacks the functional phosphatase domain. In addition, in 5ptaseIV-overexpressing cells, mature Gag relocated from the PM to CD63-positive compartments. Expression of Arf6/Q67L also reduced virus release efficiency. In this case, Gag was retargeted to newly induced PI(4,5)P₂-enriched vesicles. These results support the model that PI(4,5)P₂ promotes or stabilizes the binding of Gag to the PM by interacting with Gag.

Recently, two studies addressed the question of whether Gag interacts with PI(4,5)P₂ (75, 78). One study examined the accessibility of lysines after Gag is mixed with PI(4,5)P₂ (78). This identified two lysines at MA residues 29 and 31 as potential PI(4,5)P₂-interacting amino acids. Interestingly, we have observed previously that the mutations at these MA residues relocate Gag from the PM to compartments positive for late endosome markers in HeLa cells (58, 61). The other group determined the structure of a complex between MA and PI(4,5)P₂ using nuclear magnetic resonance (NMR) (75). As is often observed for interactions between PI(4,5)P₂ and other proteins, in this structure, the inositol head group of PI(4,5)P₂ was in direct contact with several basic residues in MA, although Lys 29 and 31 were not found to be involved. Strikingly, in addition to the head group, the 2' fatty acid chain of PI(4,5)P₂ also binds to MA at a hydrophobic cleft. Furthermore, binding of PI(4,5)P₂ to MA increased the exposure of the myristate moiety. Based on these findings, the authors proposed that PI(4,5)P₂ not only acts as a membrane anchor for Gag but also as a trigger for myristate exposure. Altogether, results from these studies are consistent with the model that Gag binds PI(4,5)P₂ directly during virus assembly.

However, two major caveats apply to these studies. First, because all the components need to be soluble in the approaches used, both studies used water-soluble PI(4,5)P₂ with short acyl chains, which does not accurately represent the natural PI(4,5)P₂ found in the cells. Secondly, both studies used Gag derivatives that are unable to drive virus assembly in cells: isolated MA and MACA were used in the NMR study, and nonmyristoylated Gag was analyzed in the lysine accessibility experiments. Thus, it remains to be determined whether the Gag-PI(4,5)P₂ interaction occurs in a physiologically relevant environment.

In this study, we analyzed the overall membrane-binding ability of Gag in control and PI(4,5)P₂-depleted cells using microscopy and an equilibrium flotation assay. The data indi-

cate that PI(4,5)P₂ is important for general membrane binding as well as PM localization, supporting the hypothesis that Gag and PI(4,5)P₂ interact. To determine whether Gag interacts with PI(4,5)P₂ in a more defined system, we developed an in vitro liposome-binding assay in which full-length myristoylated Gag and PI(4,5)P₂ with natural-length acyl chains were used. Using this assay, we analyzed both WT and MA domain mutants for their ability to bind liposomes with varied amounts of PI(4,5)P₂. Our results demonstrate that full-length myristoylated Gag binds membrane-associated PI(4,5)P₂ and that this interaction mediated by the MA basic amino acids is important for Gag membrane binding. We also demonstrate that efficient Gag membrane binding and Gag localization to the PM are separable processes that each require cellular PI(4,5)P₂.

MATERIALS AND METHODS

Cells and plasmids. HeLa cells were cultured and maintained as described previously (23). The HIV-1 molecular clone pNL4-3 was described previously (1). Molecular clones encoding Gag-Venus (pNL4-3/GagVenus) and Gag-monomeric red fluorescent protein (mRFP1) (pNL4-3/GagmRFP) were constructed by replacing the SphI-SalI region of pNL4-3 with the corresponding regions of pHIV-1-GagYFP3M and pHIV-1-GagmRFP, respectively. Both pHIV-1-GagYFP3M and pHIV-1-GagmRFP were derived from pON-HIG (73) and express the full-length Gag fused to a glycine-rich hinge (PGISGGGG GILD) and a fluorescent protein. Additionally, these two constructs encode *tat*, *rev*, and *nef* and contain a large deletion in *pol* and silent mutations that destroy the *gag-pol* frameshift site without changing the protein sequence of Gag. The fluorescent protein gene vYFP3M harbors mutations that enhance the monomeric property of the encoded protein (A206K, L221K, and F223R) (92). In the manuscript, this Venus yellow fluorescent protein (YFP) derivative is referred to as Venus for simplicity. The 5ptase expression plasmid pcDNA4TO/Myc5ptaseIV (38) and the Δ 1 mutant (55) were previously described. The plasmids expressing 5ptaseIV under the control of the HIV-1 long terminal repeat promoter in the presence of Tat (pHIV-Myc5ptaseIV and pHIV-Myc5ptaseIV Δ 1) were made by replacing the Lck gene within pHIVLck+ (a kind gift from K. Strebel) with sequences encoding either Myc-tagged full-length 5ptaseIV or the Δ 1 mutant, respectively, from corresponding pcDNA plasmids. Molecular clones encoding Gag derivatives in which the initiation codon or the entire MA sequence was replaced with the sequence of the N-terminal 10 amino acids from Fyn kinase [pNL4-3/Fyn(10)fullMA and pNL4-3/Fyn(10) Δ MA, respectively] were constructed using PCR mutagenesis. pGEMNLNR, the in vitro expression plasmid for Gag, was made by inserting the NarI-EcoRI fragment from pNL4-3 into the multiple cloning site of pGEM-1 (Promega Corporation). The derivatives of pGEMNLNR encoding MA mutants pGEMNLNR/1GA, pGEMNLNR/29KE/31KE, pGEMNLNR/29KT/31KT, pGEMNLNR/Fyn(10)fullMA, and pGEMNLNR/Fyn(10) Δ MA were made by replacing the Gag sequence of pGEMNLNR with those from pNL4-3 derivatives pNL4-3/1GA (24), pNL4-3/29KE/31KE (61), pNL4-3/29KT/31KT (22), pNL4-3/Fyn(10)fullMA, and pNL4-3/Fyn(10) Δ MA, respectively. pNL4-3/Fyn(10)fullMA/GagVenus and pNL4-3/Fyn(10) Δ MA/GagVenus were constructed by replacing the Gag sequence of pNL4-3/GagVenus with those from pNL4-3/Fyn(10)fullMA and pNL4-3/Fyn(10) Δ MA, respectively. A molecular clone encoding nonfunctional viral protease, pNL4-3/PR⁻, was described previously (34).

Transfection, virus release assay, and immunoblotting. Transfection, metabolic ³⁵S labeling of transfected cells, and immunoprecipitation of viral proteins using HIV immunoglobulin (HIV-Ig; AIDS Research and Reference Reagent Program) were performed as described elsewhere (23, 58, 61). Virus release efficiency was calculated as previously described (55). Immunoblotting was performed as described previously (56) using HIV-Ig, rabbit polyclonal anti-GFP (Clontech, Mountain View, CA), or rabbit polyclonal anti-DsRed (Clontech) as primary antibodies. Detection of specific signals was performed using the Super-Signal West Pico chemiluminescence detection kit (Pierce, Rockford, IL).

Immunostaining and fluorescence microscopy. Fixation and immunostaining of HIV-expressing HeLa cells were performed as described previously (55, 61). For quantitative analysis of Gag localization phenotypes, cells transfected with pNL4-3/GagVenus or its derivatives, or pNL4-3/GagmRFP, along with pHIV-Myc5ptaseIV or its Δ 1 derivative, were fixed and immunostained for the presence of 5ptase expression with rabbit anti-Myc antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Images of 10 to 15 fields were recorded using a

Nikon TE2000 microscope, and cells positive for full-length 5ptaseIV or the $\Delta 1$ mutant were evaluated for the Gag localization pattern. At least 100 5ptaseIV-positive, Gag-positive cells were examined for each condition.

Cell-based membrane-binding analysis. HeLa cells expressing WT or Fyn(10)/fullMA Gag, along with full-length 5ptaseIV or the $\Delta 1$ mutant, were pulse-labeled with [³⁵S]Met/Cys for 5 min and chased for 20 min. After sonication of labeled cells, the cell homogenates were subjected to a low-speed centrifugation to remove unbroken cells and the nucleus-associated materials as pellets. These pellets contain approximately 10% of total labeled Gag (data not shown) regardless of which Gag or 5ptaseIV constructs are expressed in the labeled cells. The postnuclear supernatants were collected and subjected to membrane flotation centrifugation as previously described (57). The top fractions containing membrane-bound materials, and also the bottom fractions containing non-membrane-bound materials, were pooled and subjected to immunoprecipitation using HIV-Ig. Labeled Gag in fractions was quantified by phosphorimager analyses as described previously (62, 63).

Liposome-binding assay. Chloroform solutions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (POPS), and L- α -phosphatidylinositol-4,5-bisphosphate (porcine brain) [PI(4,5)P₂] and powder forms of 1-stearoyl-2-arachidonoyl-sn-glycero-3-[phosphoinositol-4,5-bisphosphate] (triammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-[phosphoinositol-3,5-bisphosphate] (triammonium salt), and 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoinositol-3,4,5-trisphosphate (tetraammonium salt) were purchased from Avanti Polar Lipids Inc., Alabaster, AL. Either POPC and POPS or POPC, POPS, and one of the phosphoinositides were mixed in a tube at ratios described in the figure legends, and the chloroform was evaporated using a nitrogen stream. The dried lipids (total, 730 μ g) were resuspended in 50 μ l of cold 20 mM HEPES buffer (HB; pH 7.0), sonicated for 30 min in a water bath sonicator at 4°C, and further incubated in a shaker at 4°C overnight (12, 45, 46).

In vitro translation of Gag was performed using the TNT SP6 coupled reticulocyte lysate system (Promega Corporation, Madison, WI). Twenty five μ l of a reaction mixture that contained 12.5 μ l of reticulocyte lysate was prepared with [³⁵S]methionine-cysteine as instructed by the manufacturer and incubated for 30 min at 30°C, 5 μ l of the liposome suspension (total 73 μ g of lipids) prepared as above was added, and the mixture was further incubated for 90 min in the presence of liposomes.

Subsequently, the reaction mixture was diluted to 200 μ l using HB and mixed with 1 ml of HB containing 85.5% sucrose in an ultracentrifuge tube. This mixture was then layered with 2.8 ml HB containing 65% sucrose and 1 ml HB containing 10% sucrose and centrifuged at 115,000 $\times$ g at 4°C for 16 h (Sorvall rotor AH-650; 35,000 rpm). Five 1-ml fractions were collected from the top of each tube. The top two fractions represent liposome-bound Gag, and the bottom fractions represent non-liposome-bound Gag. The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the band intensity of Gag in each fraction was quantified using a phosphorimager. The amount of labeled Gag in the top two fractions versus the total amount of labeled Gag was calculated and is shown as liposome-binding efficiency. For Fig. 4, 5, and 7, below, the relative liposome-binding efficiency for each condition was calculated in comparison to the binding efficiency of WT Gag with PI(4,5)P₂-containing liposomes.

Statistical analysis. The two-tailed Student's *t* test was performed using GraphPad Prism version 3.0cx for Macintosh (GraphPad Software, San Diego, CA). The paired *t* test was used for comparing the data within the same set of experiments. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Overexpression of 5ptaseIV relocates HIV-1 Gag to the perinuclear compartments and cytosol. In a previous study, we used a monoclonal antibody against p17 MA to show that overexpression of 5ptaseIV relocates Gag to perinuclear compartments (55). However, this antibody recognizes only mature MA and not Pr55^{Gag} (94). To determine the localization of the whole population of Gag, in this study we used Gag tagged with Venus yellow fluorescent protein (51) (GagVenus) or monomeric red fluorescent protein (9) (GagmRFP). To examine the impact of 5ptaseIV overexpression on Gag localization, HeLa cells were transfected with either GagVenus or GagmRFP, along with 5ptaseIV or its derivative lacking a

FIG. 1. Overexpression of 5ptaseIV relocates Gag from the PM to perinuclear compartments and cytosol. HeLa cells expressing GagVenus (A and B) or GagmRFP (B) along with full-length 5ptaseIV (FL) or the $\Delta 1$ derivative ($\Delta 1$) were immunostained with anti-Myc (for 5ptaseIV) and analyzed with a Nikon TE2000 microscope. The number of cells with (i) Gag localized at the PM (small arrows, gray), (ii) Gag localized predominantly at intracellular compartments (arrowheads, white), and (iii) Gag localized only in the cytosol (large arrows, black) were counted. At least 100 5ptaseIV-positive, Gag-positive cells were examined for each condition. (C) Lysates of HeLa cells transfected with pNL4-3/PR⁻, pNL4-3/GagVenus, or pNL4-3/GagmRFP were subjected to SDS-PAGE and analyzed by immunoblotting using HIV-Ig, anti-GFP, or anti-DsRed. Note that predominantly single bands were detected using antibodies against fluorescent proteins.

functional phosphatase domain (the $\Delta 1$ mutant), and analyzed with a fluorescence microscope (Fig. 1A and data not shown). In cells expressing the 5ptaseIV $\Delta 1$ mutant, a majority of GagVenus and GagmRFP showed punctate PM localization as expected for WT Gag. When full-length 5ptaseIV was expressed, however, most Gag signals were detected either in perinuclear compartments or in the cytosol. To assess the effect

of 5ptaseIV more quantitatively, cells expressing GagVenus or GagmRFP were counted and divided into three groups based on Gag localization: Gag localized to the PM, Gag predominantly in the intracellular compartments, or Gag only in the cytosol without PM or intracellular localization (Fig. 1B). The cells expressing the $\Delta 1$ mutant of 5ptaseIV had Gag localized to the PM in more than half of the cells. Very few cells showed perinuclear localization. The remaining cells displayed only hazy cytosolic localization. These Venus and mRFP signals likely represent full-length Gag tagged with the fluorescent proteins, as virtually no other protein species containing the fluorescent tags were detected by immunoblotting of cell lysates (Fig. 1C). In contrast to cells expressing 5ptaseIV $\Delta 1$, cells expressing full-length 5ptaseIV had drastically increased hazy cytosolic localization and intracellular localization, whereas cells that had PM localization were reduced to only 5% of total Gag-positive cells. These results indicate that, when cellular PI(4,5)P₂ is depleted, Gag relocalizes not only to intracellular compartments as we have shown previously (55) but also to the cytosol. These data confirm the importance of PI(4,5)P₂ in PM localization of Gag. In addition, the increase in the hazy cytosolic signal in 5ptaseIV-expressing cells suggests that PI(4,5)P₂ might also be essential for total membrane binding of Gag.

Overexpression of 5ptaseIV causes a Gag membrane-binding defect. To analyze more quantitatively whether PI(4,5)P₂ depletion impairs membrane binding of Gag, we examined the membrane binding of WT Gag in HeLa cells expressing either 5ptaseIV or the $\Delta 1$ mutant by equilibrium flotation centrifugation. The cells were pulse-labeled with [³⁵S]methionine-cysteine for 5 min and chased for 20 min, and postnuclear supernatants of cell homogenates were subjected to equilibrium flotation centrifugation. The labeled Gag in both top fractions (membrane-bound) and bottom fractions (non-membrane-bound) was immunoprecipitated with HIV-Ig and quantified by phosphorimager. We found that the membrane binding of Gag was significantly decreased when full-length 5ptaseIV was expressed compared to the $\Delta 1$ mutant of 5ptaseIV (Fig. 2A and B). These results indicate that PI(4,5)P₂ is not only important for plasma membrane localization but also contributes to general membrane binding of Gag. A large part of membrane-bound Gag still detected in cells overexpressing 5ptaseIV may be a population associated with the intracellular membranes observed in Fig. 1. Notably, we did not observe a significant reduction in the membrane binding of Fyn(10)fullMA Gag (Fig. 2A and B), a Gag derivative in which the N-terminal myristoylation site is replaced by a Fyn membrane-binding signal containing one myristoylation and two palmitoylation sites. These data indicate that addition of a strong membrane-binding signal to MA can reverse the membrane-binding defect imposed by PI(4,5)P₂ depletion.

The presence of PI(4,5)P₂ increases Gag binding to liposomes. As described above, cellular PI(4,5)P₂ depletion not only relocalized Gag from the PM to intracellular compartments but also reduced the total membrane-binding ability of Gag. These results are consistent with the hypothesis that Gag interacts with PI(4,5)P₂ directly. To test this hypothesis, we developed an in vitro system using liposomes that consist of phosphatidylcholine (PC), phosphatidylserine (PS), and varied amounts of PI(4,5)P₂. An in vitro transcription and translation-coupled reaction with rabbit reticulocyte lysate was used to

FIG. 2. Membrane binding of WT Gag is reduced in 5ptaseIV-overexpressing cells. HeLa cells expressing WT or Fyn(10)fullMA Gag along with full-length 5ptaseIV (FL) or the $\Delta 1$ mutant were pulse-labeled for 5 min and chased for 20 min. Postnuclear supernatants of cell homogenates were subjected to membrane flotation centrifugation. (A) Membrane-bound (M) and non-membrane-bound (NM) Gag was recovered by immunoprecipitation and subjected to SDS-PAGE. (B) The labeled Gag protein was quantified by phosphorimager analysis, and the membrane binding efficiency was calculated. Data from six (WT) or four [Fyn(10)fullMA] different experiments are shown as means $\pm$ standard deviations. *P* values were determined using Student's *t* test. ns, not significant; **, *P* < 0.005.

synthesize full-length myristoylated Gag in the presence of [³⁵S]methionine-cysteine. This in vitro Gag synthesis has been used successfully for studying Gag membrane binding and assembly (30, 39, 44, 68, 81, 90, 93, 94). After 30 min of Gag synthesis, the reaction mixture was mixed with liposomes and further incubated for 90 min. The liposome-bound Gag and non-liposome-bound Gag were separated by equilibrium flotation centrifugation. The amount of labeled Gag in each fraction was quantified using a phosphorimager, and the percentage of Gag bound to the liposomes versus total Gag was calculated. When the PI(4,5)P₂ concentration in the liposomes was increased gradually, the liposome-bound Gag also increased (Fig. 3A and B), suggesting that the Gag-PI(4,5)P₂ interaction mediates Gag binding to liposomes.

In the experiments described above, the ratio between PC

FIG. 3. Gag binds PI(4,5)P₂ specifically in a concentration-dependent manner. ³⁵S-labeled WT Gag was synthesized in the in vitro transcription and translation system by using reticulocyte lysates and was incubated with liposomes containing various amounts of acidic phospholipids. (A and B) PC:PS (2:1) liposomes containing various amounts of PI(4,5)P₂ were used. (C and D) Liposomes containing increased levels of PS were compared with liposomes containing 62.75 mol% PC, 30 mol% PS, and 7.25 mol% PI(4,5)P₂. (E and F) A concentration of 7.25 mol% of PI(4,5)P₂, PI(3,5)P₂, and PI(3,4,5)P₃ was compared in the context of the PS:PS (2:1) liposomes. The reaction mixtures were subjected to membrane flotation centrifugation, and five 1-ml fractions were collected from each tube. M, liposome bound; NM, not liposome bound. Fractions were subjected to SDS-PAGE followed by autoradiography (A, C, and E). The amount of labeled Gag in each fraction was quantified using a phosphorimager, and the percentage of labeled Gag in the membrane-bound fraction versus total amount of labeled Gag was calculated (B, D, and F). For panel B, data from seven (0 and 7.25 mol%), five (0.63 mol%), and four (2.1, 3.52, and 5.71 mol%) different experiments are shown as means $\pm$ standard deviations. *P* values were determined between 0% PI(4,5)P₂ and various percentages of PI(4,5)P₂ using Student's *t* test (ns, not significant; **, *P* < 0.005; ***, *P* < 0.001). In panels D and F, data from three and four different experiments, respectively, are shown (ns, not significant; *, *P* < 0.05; **, *P* < 0.01).

and PS was kept constant (2:1) while the amounts of PI(4,5)P₂ were gradually increased. Therefore, it is possible that the enhanced Gag binding to liposomes in the presence of PI(4,5)P₂ may be due to the increase in the overall negative charge of lipo-

somes, not the increase in the PI(4,5)P₂ concentration per se. To address this possibility, we analyzed Gag binding to PC-PS liposomes containing elevated levels of PS. As PI(4,5)P₂ is likely trivalent or tetravalent in the negative charge under the

FIG. 4. Myristoylation-defective mutant Gag does not bind liposomes efficiently even in the presence of a high percentage of PI(4,5)P₂. (A) WT or 1GA Gag was synthesized in the reticulocyte lysate system in the presence of $[^{35}\text{S}]$ methionine-cysteine or $[^3\text{H}]$ myristic acid. The major band (labeled Pr55^{Gag}) comigrated with Gag immunoprecipitated from lysates of HeLa cells transfected with pNL4-3/PR⁻ in a parallel experiment (data not shown). In addition to the Pr55^{Gag} band, three minor bands (asterisk) were detected in the in vitro translation reaction of WT Gag performed in the presence of the ^{35}S -labeled amino acids. Two of the minor bands that are also labeled with ^3H likely represent Gag species arising from premature termination of translation, whereas the one band labeled only with ^{35}S may be formed by internal ribosomal entry. In vitro translation reaction mixtures for 1GA Gag as well as other Gag derivatives used in this study (data not shown) contained the same minor bands with similarly low abundance. (B and C) ^{35}S -labeled WT Gag or myristoylation mutant (1GA) Gag was synthesized as for Fig. 3 and incubated with liposomes containing or not containing PI(4,5)P₂. The reaction mixtures were subjected to membrane flotation centrifugation, and fractions were analyzed as for Fig. 3 (B). The amount of labeled Gag in each fraction was quantified using a phosphorimager, and the percentage of labeled Gag in the membrane-bound fraction versus the total amount of labeled Gag was calculated (C). The relative liposome-binding efficiency

conditions tested, whereas PS is monovalent (49), we examined liposomes containing 52 or 60 mol% PS that would bear equivalent levels of the negative charge as liposomes containing 30 mol% PS and 7.25 mol% PI(4,5)P₂ used in Fig. 3A and B. Liposomes with 52 or 60 mol% PS showed increased Gag binding compared to liposomes containing 30 mol% PS (Fig. 3C and D), suggesting that electrostatic interaction between Gag and lipids does play a role in Gag-liposome binding. Nevertheless, these high concentrations of PS were insufficient to mediate the high level of Gag binding observed with liposomes containing 30 mol% PS and 7.25 mol% PI(4,5)P₂ (Fig. 3C and D). These results suggest that enhanced Gag binding to PI(4,5)P₂-containing liposomes does not simply result from the increase in the overall negative charge of liposomes.

PI(4,5)P₂ may facilitate Gag binding due to the highly concentrated negative charge of its head group. Alternatively, the configuration of phosphate residues may play a key role in the specificity of the Gag-PI(4,5)P₂ interaction. To evaluate these possibilities, we compared PI(3,5)P₂ and PI(3,4,5)P₃ with PI(4,5)P₂ at 7.25 mol% for the ability to recruit Gag to liposomes containing 30 mol% PS. We observed that PI(3,5)P₂ and PI(3,4,5)P₃ substantially enhanced Gag binding to liposomes compared to the control liposomes. However, Gag binding to PI(3,5)P₂-containing liposomes was consistently less than that to PI(4,5)P₂-containing liposomes. We did not observe a significant difference between PI(4,5)P₂ and PI(3,4,5)P₃ (Fig. 3E and F). It is of note that, using water-soluble phosphoinositides, Saad et al. observed that the isolated MA domain also binds PI(4,5)P₂ and PI(3,4,5)P₃ but not PI(3,5)P₂ (75). These data suggest that not only the number but also the position of phosphates on the inositol head group affect the Gag-phosphoinositide interaction. Altogether, these results demonstrate that there is an interaction between the head group of PI(4,5)P₂ and full-length Gag and that this interaction facilitates membrane binding of Gag.

To confirm that the observed Gag binding to liposomes reflects the Gag-membrane interaction in cells, we also examined, using a liposome-binding assay, a nonmyristoylated Gag (1GA) that was previously shown to be defective in membrane binding in cell-based assays (8, 26, 57, 65, 93). As shown in Fig. 4A, WT but not 1GA Gag was myristoylated in the in vitro translation system we used. The 1GA mutant binds liposomes inefficiently even in the presence of 7.25 mol% PI(4,5)P₂ (Fig. 4B and C). These results indicate that myristoylation is required for efficient Gag-liposome interaction. These results further demonstrate that, unlike assays using water-soluble PI(4,5)P₂, the liposome-binding assay developed here is suited for examining the interaction between Gag and PI(4,5)P₂ in the context of membrane binding.

MA mediates the Gag interaction with PI(4,5)P₂. MA was shown previously to be important for both membrane binding and PM localization (2, 21, 83). To assess the importance of MA in the interaction with PI(4,5)P₂ in a liposome-binding

for each condition was calculated in comparison to the binding efficiency of WT Gag with PI(4,5)P₂-containing liposomes. Data from three different experiments are shown as means $\pm$ standard deviations. *P* values were determined using Student's *t* test. ***, *P* < 0.001.

FIG. 5. Addition of an efficient membrane-binding sequence does not eliminate the requirement for PI(4,5)P₂ in Gag liposome binding. (A) Schematic representation of Gag derivatives with the Fyn N-terminal sequence. Myristoylation (m) and palmitoylation (palm) sites are shown. (B) ³⁵S-labeled WT Gag, Fyn(10)fullMA Gag, and Fyn(10)ΔMA Gag were synthesized and incubated with liposomes as for Fig. 4. The reaction mixtures were subjected to membrane flotation centrifugation, and the fractions were analyzed as for Fig. 3. (C) The amount of labeled Gag in each fraction was quantified using a phosphorimager, and statistical analysis was performed as for Fig. 4. Data from four different experiments are shown as means $\pm$ standard deviations. *P* values were determined using Student's *t* test. ***, *P* < 0.001; *, *P* < 0.05; ns, not significant.

assay, we sought to delete the entire MA sequence. Since such MA deletion removes the site for myristoylation that is indispensable for membrane binding, we instead used a Gag derivative that lacks the entire MA sequence but contains the N-terminal 10 amino acids of the Fyn kinase, Fyn(10)ΔMA Gag (Fig. 5A). This Fyn-derived sequence is myristoylated and dually palmitoylated, thereby providing this Gag derivative with a high membrane affinity. As a control, we used Fyn(10)fullMA Gag (Fig. 5A), which retains the entire MA sequence following the Fyn-derived peptide. As shown in Fig. 5B and C, liposome binding of Fyn(10)fullMA Gag was still augmented by PI(4,5)P₂ as observed for WT Gag. However, when MA was deleted, as in Fyn(10)ΔMA Gag, Gag bound both control and PI(4,5)P₂-containing liposomes with similar efficiencies. These results demonstrate that MA confers dependence on PI(4,5)P₂ for liposome binding.

PM localization and virus release efficiency of Fyn(10)fullMA Gag are still sensitive to 5ptaseIV overexpression. In contrast to the data obtained from the liposome-binding assay, in the cell-based membrane-binding analysis, Fyn(10)fullMA Gag did not show any significant membrane-binding defect upon PI(4,5)P₂ depletion (Fig. 2). In the case of WT Gag, 5ptaseIV alters Gag localization to the PM in addition to reducing membrane binding. To investigate whether the PI(4,5)P₂ dependence of Fyn(10)fullMA Gag for liposome binding is manifested as a defect in the PM localization, we analyzed the effect of 5ptaseIV overexpression on localization of Fyn(10)fullMA GagVenus, as in Fig. 1. In 5ptaseIVΔ1-expressing cells, Fyn(10)fullMA GagVenus was predominantly localized on the PM. In cells overexpressing full-length 5ptaseIV, however, we observed that a majority of Fyn(10)fullMA GagVenus was localized to intracellular compartments (Fig. 6A and B). These results indicate that, even though general membrane binding of Fyn(10)fullMA Gag is not affected in cells (Fig. 2), PM localization of this Gag derivative still requires cellular PI(4,5)P₂. To examine whether the altered Gag localization affects virus particle production, we examined the virus release efficiency of Fyn(10)fullMA Gag in 5ptaseIV-overexpressing cells. HeLa cells were transfected with a molecular clone encoding WT Gag or Fyn(10)fullMA Gag, along with an expression vector for either 5ptaseIV or the Δ1 mutant. The cell and viral lysates were immunoprecipitated with HIV-Ig after metabolic labeling, and the virus release efficiency was calculated. Consistent with the altered localization of Gag, the virus release efficiency of Fyn(10)fullMA Gag as well as WT Gag was reduced when the cells expressed 5ptaseIV. These results indicate that cellular PI(4,5)P₂ is required separately both for efficient membrane binding and PM localization of WT Gag.

The liposome-binding assay showed no difference in binding of Fyn(10)ΔMA Gag to control and PI(4,5)P₂-containing liposomes (Fig. 5). Therefore, we predicted that Gag localization and virus particle production of Fyn(10)ΔMA Gag would be insensitive to 5ptaseIV overexpression, in contrast to Fyn(10)fullMA. Venus-tagged Fyn(10)ΔMA Gag localized not only to the PM but also to intracellular vesicles in a majority of cells expressing 5ptaseIV Δ1 (Fig. 6A and B). This promiscuous localization of Fyn(10)ΔMA Gag, which was also observed previously for a similar Gag derivative lacking MA (71), underscores the importance of MA in specific Gag localization to the PM. Notably, overexpression of full-length 5ptaseIV did not cause a readily detectable qualitative difference in Gag localization (Fig. 6A and B). Nevertheless, 5ptaseIV overexpression significantly reduced Fyn(10)ΔMA Gag virus release efficiency (Fig. 6C and D). We are currently investigating the possibility that cellular PI(4,5)P₂ might play an additional role in HIV-1 particle production.

Matrix basic domain mutants display a PI(4,5)P₂ binding defect in vitro. As described in the introduction, MA contains a basic amino acid cluster that was previously shown to play an important role in plasma membrane localization of Gag (24, 31, 61, 91). To see if the basic residues in this cluster facilitate the MA-PI(4,5)P₂ interaction, we tested mutants in which lysines were replaced with either glutamic acid (29KE/31KE) or threonine (29KT/31KT) at MA residues 29 and 31, using the in vitro liposome-binding assay. Full-length Gag with WT MA or with MA mutations 29KE/31KE or 29KT/31KT was synthe-

FIG. 6. Virus release efficiency and PM localization of Fyn(10)fullMA Gag are still sensitive to PI(4,5)P₂ depletion in cells. (A) HeLa cells expressing WT, Fyn(10)fullMA, or Fyn(10) Δ MA GagVenus along with full-length 5ptaseIV (FL) or the Δ 1 mutant (Δ 1) were immunostained with anti-Myc antibody and analyzed with a Nikon TE2000 microscope. (B) The number of cells with (i) Gag localized at the PM (small arrows, gray), (ii) Gag localized predominantly at intracellular compartments (arrowheads, white), and (iii) Gag localized only in the cytosol (large arrows, black) were counted as for Fig. 1. Note that for cells expressing Fyn(10) Δ MA Gag, a majority of cells showing Gag signals at the intracellular vesicles

FIG. 7. The basic domain of MA is important for efficient Gag-PI(4,5)P₂ interaction. ³⁵S-labeled WT Gag and basic domain mutants (29KE/31KE or 29KT/31KT) were synthesized and incubated with liposomes as for Fig. 4. (A) The reaction mixtures were subjected to membrane flotation centrifugation, and the fractions were analyzed as for Fig. 3. (B) The amount of labeled Gag in each fraction was quantified using a phosphorimager, and the statistical analysis was performed as for Fig. 4. Data from three different experiments are shown as means $\pm$ standard deviations. *P* values were determined using Student's *t* test. **, *P* < 0.005; *, *P* < 0.05; ns, not significant.

sized using an in vitro translation system and was allowed to interact with the control liposomes or liposomes containing PI(4,5)P₂. There was a 2.5-fold decrease in binding of both mutants to PI(4,5)P₂-containing liposomes compared to WT Gag (Fig. 7A and B). Both of these Gag mutants were previously shown to localize in intracellular vesicles using immunofluorescence microscopy (58, 61). These results indicate that the efficient interaction of Gag with PI(4,5)P₂ requires basic amino acids in the MA domain, especially residues at positions 29 and 31, and suggest that this interaction is important for the plasma membrane localization of Gag.

DISCUSSION

We have shown previously that PI(4,5)P₂ is essential for plasma membrane localization of HIV-1 Gag. In this study, using microscopy (Fig. 1) and biochemical methods (Fig. 2), we

observed that PI(4,5)P₂ is not only important for PM localization but also contributes to overall membrane binding of HIV-1 Gag. Using a liposome-binding assay, we further demonstrated that the MA domain of Gag and PI(4,5)P₂ interact with each other. Recently, using different approaches, two groups (75, 78) reported evidence supporting a possibility that MA and PI(4,5)P₂ interact directly. By solving the structure of the MA-PI(4,5)P₂ complex by NMR, one of these studies provided detailed information on the molecular contacts between MA and PI(4,5)P₂ at a high resolution (75). As described in the introduction, however, whether the interaction observed in these studies was physiological was unknown. In the liposome-binding assay described in this report, we used membrane-associated PI(4,5)P₂ with natural-length acyl chains. Furthermore, the Gag synthesized by the in vitro translation system is full-length, myristoylated Gag precursor that is capable of driving HIV assembly in cells. Thus, this assay allowed us to examine the interaction between Gag and PI(4,5)P₂ using components relevant to virus assembly in cells. Using this assay, we observed that only when PI(4,5)P₂ is present does Gag bind efficiently to the liposomes. Altogether, these results demonstrate that the full-length HIV-1 Gag indeed interacts with membrane-associated PI(4,5)P₂ and that this interaction contributes to efficient Gag membrane binding in cells.

Using the liposome-binding assay, we observed an increase in the level of Gag binding when more PI(4,5)P₂ was present in the liposomes. Though there was negligible binding to liposomes containing up to 2 mol% PI(4,5)P₂, increasing the PI(4,5)P₂ level to 3.5 mol% or higher enhanced Gag binding significantly. It has been shown that 5 mol% PI(4,5)P₂ is required for liposome binding of cellular PI(4,5)P₂-binding proteins, including ezrin, myosin VI, and gelsolin (36, 54, 82). As PI(4,5)P₂ is thought to be present at 1 to 1.5 mol% of total PM lipid in cells (11, 20, 28), one might pose a question that the binding seen in the presence of higher levels of this lipid may not be physiologically relevant. However, several points bear consideration. First, the PI(4,5)P₂ concentration in the PM has been determined using erythrocytes (11, 20, 28). These cells have a unique cytoskeleton structure (5, 27, 86) and lack the regular endocytosis function (77). As actin cytoskeleton rearrangement and endocytosis are two major PI(4,5)P₂-regulated cellular functions in other cell types (13, 47, 49, 85), PI(4,5)P₂ levels in erythrocytes may differ from those in other cell types. Second, it is important to point out that liposomes used in this assay are not completely representative of the PM, especially with regard to membrane microdomain structures. They lack cholesterol and other lipids that may form lipid rafts, which have been shown to be important for HIV-1 assembly in cells (33, 42, 43, 53, 59, 60, 62), particularly at the step of Gag membrane binding (62). Furthermore, it has been proposed

(category 2) also displayed Gag signals at the plasma membrane. (C) HeLa cells expressing WT, Fyn(10)fullMA, or Fyn(10) Δ MA Gag along with full-length 5ptaseIV (FL) or Δ 1 mutant (Δ 1) were metabolically labeled for 120 min. Cell- and virus-associated Gag were recovered by immunoprecipitation and analyzed by SDS-PAGE. (D) Signal intensity of labeled Gag was quantified by phosphorimager analysis. Virus release efficiency was calculated as the amount of virus-associated Gag as a fraction of total Gag synthesized during the labeling period and normalized to the virus release efficiency in 5ptaseIV Δ 1-expressing cultures. The average virus release efficiencies by cells expressing WT, Fyn(10)fullMA, and Fyn(10) Δ MA Gag along with 5ptaseIV Δ 1 were 15.6%, 27%, and 21.3%, respectively. Data from four different experiments are shown as means $\pm$ standard deviations. *P* values were determined using Student's *t* test. ***, *P* < 0.001; **, *P* < 0.005; *, *P* < 0.05.

that PI(4,5)P₂ may be sequestered to form microdomains and induce raft-like structures (25, 40, 48), which would lead to higher local PI(4,5)P₂ concentrations. Finally, in cells, PI(3,4,5)P₃ might also support the PM binding of Gag. A recent paper has shown that proteins with polybasic clusters dissociated from the PM only when both PI(4,5)P₂ and PI(3,4,5)P₃, but not PI(4,5)P₂ alone, were depleted (29). This may also be the case for HIV-1 Gag, which is sensitive to 5ptaseIV overexpression. Since 5ptaseIV removes the D5-phosphate not only from PI(4,5)P₂, but also from PI(3,4,5)P₃ (38), the defect observed in 5ptaseIV-expressing cells could be due to dephosphorylation of both phosphoinositides. Consistent with this possibility, the NMR study (75) showed that HIV-1 MA bound soluble PI(4,5)P₂ and PI(3,4,5)P₃ with equal affinity. A substantial interaction was also observed between full-length Gag and membrane-associated PI(3,4,5)P₃ in the current study. Thus, PI(3,4,5)P₃ might also contribute to the membrane binding of Gag along with PI(4,5)P₂ in cells.

Even if PI(4,5)P₂ is not available for Gag binding, addition of the efficient membrane-binding signal derived from Fyn kinase to the N terminus of Gag would be expected to allow Gag to bind any liposomes with equal efficiency. However, when Fyn(10)fullMA Gag was analyzed using the liposome-binding assay, binding of this Gag derivative to control liposomes was weak, and the presence of PI(4,5)P₂ augmented the binding severalfold (Fig. 5). Notably, binding to control liposomes was enhanced when MA was removed, as observed with Fyn(10)ΔMA Gag, which bound control and PI(4,5)P₂-containing liposomes with a similar efficiency (Fig. 5). Thus, MA renders Gag-liposome binding dependent on PI(4,5)P₂. We speculate, based on these data and a recent report (67), that MA in the context of full-length Gag might have a negative effect on liposome binding, which may be eliminated by the MA-PI(4,5)P₂ interaction. In the absence of MA, this negative effect no longer exists, hence Fyn(10)ΔMA Gag binds both control and PI(4,5)P₂-containing liposomes efficiently. In contrast to the liposome-binding assay, where there is only a limited number of membrane components, the cell-based assay showed that Fyn(10)fullMA Gag binds membranes well, even in 5ptaseIV-expressing cells (Fig. 2). This difference may be due to cellular components that are absent from liposomes. These factors might reduce the suppressive effect imposed by MA to a level low enough for the Fyn sequence to overcome. This difference illustrates that the defined nature of lipid membrane used in the liposome-binding assay allows the contribution of PI(4,5)P₂ to be evaluated in the absence of other membrane factors that, in the cell, can contribute to Gag membrane binding.

In the liposome-binding assay described in this report, very little Gag associated with liposomes in the absence of PI(4,5)P₂, even though more than 30 mol% of the liposomes consisted of another acidic lipid, PS. In contrast, a number of groups have observed that PS can support liposome binding of Gag in the absence of PI(4,5)P₂ (4, 6, 14, 19, 76, 93). Some studies used a very high level of PS (e.g., PC:PS at a 1:2 ratio), which may account for the efficient PS-mediated membrane binding (discussed below). Dalton et al. showed recently, however, that myristoylated HIV-1 MA efficiently binds to PC:PS (2:1) liposomes (14). This discrepancy can be accounted for by differences in experimental systems. One such difference is that

Dalton et al. observed efficient binding of myristoylated MA or its derivative fused with the CA C-terminal domain (MA-CACTD) to PS using much higher concentrations of the proteins than obtained in our assay. In this way, they were able to measure a weak interaction between MA and PS. In addition, a high concentration of Gag likely facilitates Gag multimerization, thereby increasing its avidity for membranes. Indeed, Dalton et al. found that dimerization of MA-CACTD significantly enhances binding to PS-containing liposomes. Whether Gag forms multimers under the conditions we used in our assay is currently unknown. Second, the use of MA (or MA-CACTD) versus full-length Gag may also contribute to the difference in the PS interaction. It is possible that the structure of isolated MA and the MA domain of full-length Gag may not be identical and that such conformational differences may affect the affinity for PS. It was recently proposed that full-length Gag adopts a folded conformation in which MA and NC are in close proximity (15). In the context of full-length Gag, such a folded conformation may restrict the access of PS to the MA basic domain, which is freely accessible for PS in isolated MA or MA-CACTD. Lastly, yet another important difference is that, in our experiments, components of rabbit reticulocyte lysate used for synthesizing Gag were present during incubation of Gag with liposomes, whereas Dalton et al. analyzed liposome binding of purified proteins in the absence of other cellular components. Thus, in our system, negatively charged molecules, such as IP6 and RNA, or positively charged molecules, such as polyamines, both of which are present in mammalian cells, may interfere with the weak electrostatic interaction between Gag and PS. Indeed, both IP6 and RNA were previously shown to bind MA (16, 64). Altogether, we consider our *in vitro* assay as an approach complementary to existing ones in that it focuses on relatively strong interactions between Gag and membrane lipids, which overcome the interference by charged molecules present in mammalian cells.

Even in our liposome-binding assay, elevating the fraction of PS from 30 mol% to 52 or 60 mol% augmented Gag binding (Fig. 3C and D). However, liposomes containing 7.25 mol% PI(4,5)P₂ along with 30 mol% PS allowed more Gag binding than those containing 60 mol% PS (Fig. 3C and D), suggesting that the high charge density of PI(4,5)P₂ plays a key role in Gag binding. These data are largely consistent with the computational work of Murray et al. (50), in which an electrostatic interaction was shown to determine the MA-membrane interaction. In this modeling study, when PI(4,5)P₂ was present in the lipid bilayer, it significantly increased the electrostatic potential due to its high negative charge density, thus enhancing membrane binding of Gag (50). Compared with PI(4,5)P₂, however, PI(3,5)P₂ was consistently less efficient in facilitating Gag-liposome binding (Fig. 3E and F). These findings, in agreement with the NMR study (75), suggest that, in addition to a high density of negative charge, a specific configuration of phosphates on the inositol head group may play a role in the preferential interaction of Gag with PI(4,5)P₂.

Previously, we have observed that mutations at MA residues 29 and 31 within the highly basic sequence relocalized Gag from the PM to compartments positive for late endosome markers in HeLa and T cells. Using the liposome-binding assay, we observed that the 29KE/31KE Gag interacts less effi-

ciently with PI(4,5)P₂-containing liposomes than WT Gag. This is not simply due to a drastic change of charge from positive to negative, since the 29KT/31KT Gag, in which the same lysine residues are replaced with neutral amino acids, showed a similar level of reduction in binding to PI(4,5)P₂-containing liposomes. It is also unlikely that these mutations caused a major conformational change that in turn resulted in the reduced binding to PI(4,5)P₂-containing liposomes, as Gag derivatives containing these amino acid changes are capable of forming virus particles with apparently a normal morphology at intracellular vesicles (24, 61). Interestingly, Shkriabai et al. observed that the same lysine residues were masked from solvent when water-soluble PI(4,5)P₂ was added to nonmyristoylated but full-length Gag (78). In contrast, the NMR study performed using isolated MA in complex with PI(4,5)P₂ did not show an interaction of MA residues 29 and 31 with PI(4,5)P₂ (75). This difference in the residues involved in the PI(4,5)P₂ interaction may be due to differences in the conformation of the MA domain of Gag versus mature MA. We are currently examining the role of CA and NC domains in PI(4,5)P₂-dependent liposome binding. These experiments will also likely provide insights into the ability of PS to support the MA-liposome binding discussed above. It is of note that the binding of the mutants 29KE/31KE and 29KT/31KT to PI(4,5)P₂-containing liposomes was not totally abolished, suggesting that there might be other basic amino acids playing a role in the Gag-PI(4,5)P₂ interaction. The analysis of other basic MA amino acid mutants for their ability in binding PI(4,5)P₂ is also ongoing in our laboratory.

One possible caveat of the liposome-binding assay developed and used in this report might be the nonuniformity of liposomes. PI(4,5)P₂ is not as soluble in chloroform as other lipids (87). Therefore, in the procedure we used to prepare liposomes, this differential solubility may lead to nonuniform mixing of lipids, which may in turn result in variable percentages of PI(4,5)P₂ in each individual liposome. In addition, as discussed above, reticulocyte lysates present in the assay mixture likely contain molecules that modulate the Gag-PI(4,5)P₂ interaction. Therefore, this assay should not be used to determine the affinity of Gag for PI(4,5)P₂. Nonetheless, the assay described in this paper will be valuable in investigating requirements for the Gag-phosphoinositide interaction.

In summary, we have shown that full-length Gag binds to PI(4,5)P₂ associated with membranes under conditions relevant for virus assembly in cells, and this MA-mediated interaction is important for efficient Gag membrane binding. We have also confirmed that, apart from membrane binding, cellular PI(4,5)P₂ is also important for PM localization of Gag. Due to the continuous need for new drugs against HIV-1, it is important to look at interactions between viral proteins and cellular factors like PI(4,5)P₂ as potential drug targets. Although manipulation of cellular PI(4,5)P₂ may not be a viable antiretroviral strategy because of the physiological importance of this lipid in cells, inhibitors that block Gag binding to PI(4,5)P₂ could perhaps be developed. Further characterization of the Gag-PI(4,5)P₂ interaction using the liposome-binding assay established in this study will likely provide groundwork for this goal.

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REFERENCES

- Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**:284–291.
- Adamson, C. S., and I. M. Jones. 2004. The molecular basis of HIV capsid assembly—five years of progress. *Rev. Med. Virol.* **14**:107–121.
- Aikawa, Y., and T. F. Martin. 2003. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. *J. Cell Biol.* **162**:647–659.
- Alfadhli, A., D. Huseby, E. Kapit, D. Colman, and E. Barklis. 2007. Human immunodeficiency virus type 1 matrix protein assembles on membranes as a hexamer. *J. Virol.* **81**:1472–1478.
- Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* **54**:273–304.
- Bouamr, F., S. Scarlata, and C. Carter. 2003. Role of myristylation in HIV-1 Gag assembly. *Biochemistry* **42**:6408–6417.
- Brown, F. D., A. L. Rozelle, H. L. Yin, T. Balla, and J. G. Donaldson. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* **154**:1007–1017.
- Bryant, M., and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87**:523–527.
- Campbell, R. E., O. Tour, A. E. Palmer, P. A. Steinbach, G. S. Baird, D. A. Zacharias, and R. Y. Tsien. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**:7877–7882.
- Campbell, S., R. J. Fisher, E. M. Towler, S. Fox, H. J. Issaq, T. Wolfe, L. R. Phillips, and A. Rein. 2001. Modulation of HIV-like particle assembly in vitro by inositol phosphates. *Proc. Natl. Acad. Sci. USA* **98**:10875–10879.
- Christensen, S. 1986. Removal of haem from lipids extracted from intact erythrocytes with particular reference to polyphosphoinositides. *Biochem. J.* **233**:921–924.
- Chung, S. H., W. J. Song, K. Kim, J. J. Bednarski, J. Chen, G. D. Prestwich, and R. W. Holz. 1998. The C2 domains of Rabphilin3A specifically bind phosphatidylinositol 4,5-bisphosphate containing vesicles in a Ca²⁺-dependent manner. In vitro characteristics and possible significance. *J. Biol. Chem.* **273**:10240–10248.
- Czech, M. P. 2000. PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**:603–606.
- Dalton, A. K., D. Ako-Adjei, P. S. Murray, D. Murray, and V. M. Vogt. 2007. Electrostatic interactions drive membrane association of the human immunodeficiency virus type 1 Gag MA domain. *J. Virol.* **81**:6434–6445.
- Datta, S. A., J. E. Curtis, W. Ratcliff, P. K. Clark, R. M. Crist, J. Lebowitz, S. Krueger, and A. Rein. 2007. Conformation of the HIV-1 Gag protein in solution. *J. Mol. Biol.* **365**:812–824.
- Datta, S. A., Z. Zhao, P. K. Clark, S. Tarasov, J. N. Alexandratos, S. J. Campbell, M. Kvaratskhelia, J. Lebowitz, and A. Rein. 2007. Interactions between HIV-1 Gag molecules in solution: an inositol phosphate-mediated switch. *J. Mol. Biol.* **365**:799–811.
- Deneka, M., A. Pelchen-Matthews, R. Byland, E. Ruiz-Mateos, and M. Marsh. 2007. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J. Cell Biol.* **177**:329–341.
- DiNitto, J. P., T. C. Cronin, and D. G. Lambright. 2003. Membrane recognition and targeting by lipid-binding domains. *Sci. STKE* **2003**:re16.
- Ehrlich, L. S., S. Fong, S. Scarlata, G. Zybarch, and C. Carter. 1996. Partitioning of HIV-1 Gag and Gag-related proteins to membranes. *Biochemistry* **35**:3933–3943.
- Ferrell, J. E., Jr., and W. H. Huettis. 1984. Phosphoinositide metabolism and the morphology of human erythrocytes. *J. Cell Biol.* **98**:1992–1998.
- Freed, E. O. 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* **251**:1–15.
- Freed, E. O., G. Englund, and M. A. Martin. 1995. Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J. Virol.* **69**:3949–3954.
- Freed, E. O., and M. A. Martin. 1994. Evidence for a functional interaction

- between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. *J. Virol.* **68**:2503–2512.
24. Freed, E. O., J. M. Orenstein, A. J. Buckler-White, and M. A. Martin. 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.* **68**:5311–5320.
 25. Golebiewska, U., A. Gambhir, G. Hangyas-Mihalyne, I. Zaitseva, J. Radler, and S. McLaughlin. 2006. Membrane-bound basic peptides sequester multivalent (PIP2), but not monovalent (PS), acidic lipids. *Biophys. J.* **91**:588–599.
 26. Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**:5781–5785.
 27. Gratzer, W. B. 1981. The red cell membrane and its cytoskeleton. *Biochem. J.* **198**:1–8.
 28. Hagelberg, C., and D. Allan. 1990. Restricted diffusion of integral membrane proteins and polyphosphoinositides leads to their depletion in microvesicles released from human erythrocytes. *Biochem. J.* **271**:831–834.
 29. Heo, W. D., T. Inoue, W. S. Park, M. L. Kim, B. O. Park, T. J. Wandless, and T. Meyer. 2006. PI(3,4,5)P₃ and PI(4,5)P₂ lipids target proteins with polybasic clusters to the plasma membrane. *Science* **314**:1458–1461.
 30. Hermida-Matsumoto, L., and M. D. Resh. 1999. Human immunodeficiency virus type 1 protease triggers a myristoyl switch that modulates membrane binding of Pr55^{gag} and p17MA. *J. Virol.* **73**:1902–1908.
 31. Hermida-Matsumoto, L., and M. D. Resh. 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* **74**:8670–8679.
 32. Hill, C. P., D. Worthylake, D. P. Bancroft, A. M. Christensen, and W. I. Sundquist. 1996. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc. Natl. Acad. Sci. USA* **93**:3099–3104.
 33. Holm, K., K. Weclawicz, R. Hewson, and M. Suomalainen. 2003. Human immunodeficiency virus type 1 assembly and lipid rafts: Pr55^{gag} associates with membrane domains that are largely resistant to Brij98 but sensitive to Triton X-100. *J. Virol.* **77**:4805–4817.
 34. Huang, M., J. M. Orenstein, M. A. Martin, and E. O. Freed. 1995. p6^{Gag} is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J. Virol.* **69**:6810–6818.
 35. Hurley, J. H., and T. Meyer. 2001. Subcellular targeting by membrane lipids. *Curr. Opin. Cell Biol.* **13**:146–152.
 36. Janmey, P. A., K. Iida, H. L. Yin, and T. P. Stossel. 1987. Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. *J. Biol. Chem.* **262**:12228–12236.
 37. Jouvenet, N., S. J. Neil, C. Bess, M. C. Johnson, C. A. Virgen, S. M. Simon, and P. D. Bieniasz. 2006. Plasma membrane is the site of productive HIV-1 particle assembly. *PLoS Biol.* **4**:e435.
 38. Kisseleva, M. V., M. P. Wilson, and P. W. Majerus. 2000. The isolation and characterization of a cDNA encoding phospholipid-specific inositol polyphosphate 5-phosphatase. *J. Biol. Chem.* **275**:20110–20116.
 39. Klein, K. C., S. J. Polyak, and J. R. Lingappa. 2004. Unique features of hepatitis C virus capsid formation revealed by de novo cell-free assembly. *J. Virol.* **78**:9257–9269.
 40. Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P₂ at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* **149**:1455–1472.
 41. Lemmon, M. A. 2003. Phosphoinositide recognition domains. *Traffic* **4**:201–213.
 42. Lindwasser, O. W., and M. D. Resh. 2001. Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. *J. Virol.* **75**:7913–7924.
 43. Lindwasser, O. W., and M. D. Resh. 2002. Myristoylation as a target for inhibiting HIV assembly: unsaturated fatty acids block viral budding. *Proc. Natl. Acad. Sci. USA* **99**:13037–13042.
 44. Lingappa, J. R., R. L. Hill, M. L. Wong, and R. S. Hegde. 1997. A multistep, ATP-dependent pathway for assembly of human immunodeficiency virus capsids in a cell-free system. *J. Cell Biol.* **136**:567–581.
 45. Low, J. A., B. Magnuson, B. Tsai, and M. J. Imperiale. 2006. Identification of gangliosides GD1b and GT1b as receptors for BK virus. *J. Virol.* **80**:1361–1366.
 46. Magnuson, B., E. K. Rainey, T. Benjamin, M. Baryshev, S. Mkrtchian, and B. Tsai. 2005. ERp29 triggers a conformational change in polyomavirus to stimulate membrane binding. *Mol. Cell* **20**:289–300.
 47. Martin, T. F. J. 2001. PI(4,5)P₂ regulation of surface membrane traffic. *Curr. Opin. Cell Biol.* **13**:493–499.
 48. McLaughlin, S., and D. Murray. 2005. Plasma membrane phosphoinositide organization by protein electrostatics. *Nature* **438**:605–611.
 49. McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP2 and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **31**:151–175.
 50. Murray, P. S., Z. Li, J. Wang, C. L. Tang, B. Honig, and D. Murray. 2005. Retroviral matrix domains share electrostatic homology: models for membrane binding function throughout the viral life cycle. *Structure (Cambridge)* **13**:1521–1531.
 51. Nagai, T., K. Ibata, E. S. Park, M. Kubota, K. Mikoshiba, and A. Miyawaki. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**:87–90.
 52. Nguyen, D. G., A. Booth, S. J. Gould, and J. E. Hildreth. 2003. Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. *J. Biol. Chem.* **278**:52347–52354.
 53. Nguyen, D. H., and J. E. Hildreth. 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74**:3264–3272.
 54. Niggli, V., C. Andreoli, C. Roy, and P. Mangeat. 1995. Identification of a phosphatidylinositol-4,5-bisphosphate-binding domain in the N-terminal region of ezrin. *FEBS Lett.* **376**:172–176.
 55. Ono, A., S. D. Ablan, S. J. Lockett, K. Nagashima, and E. O. Freed. 2004. Phosphatidylinositol(4,5)bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc. Natl. Acad. Sci. USA* **101**:14889–14894.
 56. Ono, A., D. Demirov, and E. O. Freed. 2000. Relationship between human immunodeficiency virus type 1 Gag multimerization and membrane binding. *J. Virol.* **74**:5142–5150.
 57. Ono, A., and E. O. Freed. 1999. Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. *J. Virol.* **73**:4136–4144.
 58. Ono, A., and E. O. Freed. 2004. Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. *J. Virol.* **78**:1552–1563.
 59. Ono, A., and E. O. Freed. 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc. Natl. Acad. Sci. USA* **98**:13925–13930.
 60. Ono, A., and E. O. Freed. 2005. Role of lipid rafts in virus replication. *Adv. Virus Res.* **64**:311–358.
 61. Ono, A., J. M. Orenstein, and E. O. Freed. 2000. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J. Virol.* **74**:2855–2866.
 62. Ono, A., A. A. Waheed, and E. O. Freed. 2007. Depletion of cellular cholesterol inhibits membrane binding and higher-order multimerization of human immunodeficiency virus type 1 Gag. *Virology* **360**:27–35.
 63. Ono, A., A. A. Waheed, A. Joshi, and E. O. Freed. 2005. Association of human immunodeficiency virus type 1 gag with membrane does not require highly basic sequences in the nucleocapsid: use of a novel Gag multimerization assay. *J. Virol.* **79**:14131–14140.
 64. Ott, D. E., L. V. Coren, and T. D. Gagliardi. 2005. Redundant roles for nucleocapsid and matrix RNA-binding sequences in human immunodeficiency virus type 1 assembly. *J. Virol.* **79**:13839–13847.
 65. Paillart, J. C., and H. G. Gottlinger. 1999. Opposing effects of human immunodeficiency virus type 1 matrix mutations support a myristoyl switch model of Gag membrane targeting. *J. Virol.* **73**:2604–2612.
 66. Pelchen-Matthews, A., B. Kramer, and M. Marsh. 2003. Infectious HIV-1 assembles in late endosomes in primary macrophages. *J. Cell Biol.* **162**:443–455.
 67. Perez-Caballero, D., T. Hatzioannou, J. Martin-Serrano, and P. D. Bieniasz. 2004. Human immunodeficiency virus type 1 matrix inhibits and confers cooperativity on gag precursor-membrane interactions. *J. Virol.* **78**:9560–9563.
 68. Platt, E. J., and O. K. Haffar. 1994. Characterization of human immunodeficiency virus type 1 Pr55^{gag} membrane association in a cell-free system: requirement for a C-terminal domain. *Proc. Natl. Acad. Sci. USA* **91**:4594–4598.
 69. Rao, Z., A. S. Belyaev, E. Fry, P. Roy, I. M. Jones, and D. I. Stuart. 1995. Crystal structure of SIV matrix antigen and implications for virus assembly. *Nature* **378**:743–747.
 70. Raposo, G., M. Moore, D. Innes, R. Leijendekker, A. Leigh-Brown, P. Benaroch, and H. Geuze. 2002. Human macrophages accumulate HIV-1 particles in MHC II compartments. *Traffic* **3**:718–729.
 71. Reil, H., A. Bukovsky, H. R. Gelderblom, and H. G. Gottlinger. 1998. Efficient HIV-1 replication can occur in the absence of the viral matrix protein. *EMBO J.* **17**:2699–2708.
 72. Resh, M. D. 2004. A myristoyl switch regulates membrane binding of HIV-1 Gag. *Proc. Natl. Acad. Sci. USA* **101**:417–418.
 73. Rhodes, T. D., O. Nikolaitchik, J. Chen, D. Powell, and W. S. Hu. 2005. Genetic recombination of human immunodeficiency virus type 1 in one round of viral replication: effects of genetic distance, target cells, accessory genes, and lack of high negative interference in crossover events. *J. Virol.* **79**:1666–1677.
 74. Saad, J. S., E. Loeliger, P. Luncsford, M. Liriano, J. Tai, A. Kim, J. Miller, A. Joshi, E. O. Freed, and M. F. Summers. 2007. Point mutations in the HIV-1 matrix protein turn off the myristoyl switch. *J. Mol. Biol.* **366**:574–585.
 75. Saad, J. S., J. Miller, J. Tai, A. Kim, R. H. Ghanam, and M. F. Summers. 2006. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. *Proc. Natl. Acad. Sci. USA* **103**:11364–11369.
 76. Scarlata, S., L. S. Ehrlich, and C. A. Carter. 1998. Membrane-induced

- alterations in HIV-1 Gag and matrix protein-protein interactions. *J. Mol. Biol.* **277**:161–169.
77. **Schekman, R., and S. J. Singer.** 1976. Clustering and endocytosis of membrane receptors can be induced in mature erythrocytes of neonatal but not adult humans. *Proc. Natl. Acad. Sci. USA* **73**:4075–4079.
 78. **Shkriabai, N., S. A. Datta, Z. Zhao, S. Hess, A. Rein, and M. Kvaratskhelia.** 2006. Interactions of HIV-1 Gag with assembly cofactors. *Biochemistry* **45**:4077–4083.
 79. **Simonsen, A., A. E. Wurmser, S. D. Emr, and H. Stenmark.** 2001. The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* **13**:485–492.
 80. **Spearman, P., R. Horton, L. Ratner, and I. Kuli-Zade.** 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. *J. Virol.* **71**:6582–6592.
 81. **Spearman, P., and L. Ratner.** 1996. Human immunodeficiency virus type 1 capsid formation in reticulocyte lysates. *J. Virol.* **70**:8187–8194.
 82. **Spudich, G., M. V. Chibalina, J. S. Au, S. D. Arden, F. Buss, and J. Kendrick-Jones.** 2007. Myosin VI targeting to clathrin-coated structures and dimerization is mediated by binding to Disabled-2 and PtdIns(4,5)P₂. *Nat. Cell Biol.* **9**:176–183.
 83. **Swanstrom, R., and J. W. Wills.** 1997. Synthesis, assembly, and processing of viral proteins, p. 263–334. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), *Retroviruses*. Cold Spring Harbor Laboratory Press, New York, NY.
 84. **Tang, C., E. Loeliger, P. Luncsford, I. Kinde, D. Beckett, and M. F. Summers.** 2004. Entropic switch regulates myristate exposure in the HIV-1 matrix protein. *Proc. Natl. Acad. Sci. USA* **101**:517–522.
 85. **Toker, A.** 1998. The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* **10**:254–261.
 86. **Ursitti, J. A., D. W. Pumplin, J. B. Wade, and R. J. Bloch.** 1991. Ultrastructure of the human erythrocyte cytoskeleton and its attachment to the membrane. *Cell. Motil. Cytoskeleton* **19**:227–243.
 87. **Wang, J., A. Gambhir, G. Hangyas-Mihalyne, D. Murray, U. Golebiewska, and S. McLaughlin.** 2002. Lateral sequestration of phosphatidylinositol 4,5-bisphosphate by the basic effector domain of myristoylated alanine-rich C kinase substrate is due to nonspecific electrostatic interactions. *J. Biol. Chem.* **277**:34401–34412.
 88. **Welsch, S., O. T. Keppler, A. Habermann, I. Allespach, J. Krijnse-Locker, and H. G. Krausslich.** 2007. HIV-1 buds predominantly at the plasma membrane of primary human macrophages. *PLoS Pathog.* **3**:e36.
 89. **Wenk, M. R., and P. De Camilli.** 2004. Inaugural article. Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. *Proc. Natl. Acad. Sci. USA* **101**:8262–8269.
 90. **Yang, L., and L. Ratner.** 2002. Interaction of HIV-1 gag and membranes in a cell-free system. *Virology* **302**:164–173.
 91. **Yuan, X., X. Yu, T. H. Lee, and M. Essex.** 1993. Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. *J. Virol.* **67**:6387–6394.
 92. **Zacharias, D. A., J. D. Violin, A. C. Newton, and R. Y. Tsien.** 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**:913–916.
 93. **Zhou, W., L. J. Parent, J. W. Wills, and M. D. Resh.** 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J. Virol.* **68**:2556–2569.
 94. **Zhou, W., and M. D. Resh.** 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J. Virol.* **70**:8540–8548.