Interactions of HIV-1 Gag with Assembly Cofactors†

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ABSTRACT: HIV-1 Gag is the only protein required for retroviral particle assembly. There is evidence suggesting that phosphatidylinositol phosphate and nucleic acid are essential for viruslike particle assembly. To elucidate structural foundations of interactions of HIV-1 Gag with the assembly cofactors $PI(4,5)P_2$ and RNA, we employed mass spectrometric protein footprinting. In particular, the NHS-biotin modification approach was used to identify the lysine residues that are exposed to the solvent in free Gag and are protected from biotinylation by direct protein−ligand or protein−protein contacts in Gag complexes with $PI(4,5)P_2$ and/or RNA. Of 21 surface lysines readily modified in free Gag, only K30 and K32, located in the matrix domain, were strongly protected in the Gag−$PI(4,5)P_2$ complex. Nucleic acid also protected these lysines, but only at significantly higher concentrations. In contrast, nucleic acids and not $PI(4,5)P_2$ exhibited strong protection of two nucleocapsid domain residues: K391 and K424. In addition, K314, located in the capsid domain, was specifically protected only in the presence of both $PI(4,5)P_2$ and nucleic acid. We suggest that concerted binding of $PI(4,5)P_2$ and nucleic acid to the matrix and nucleocapsid domains, respectively, promotes protein−protein interactions involving capsid domains. These protein−protein interactions must be involved in virus particle assembly.

HIV-1 particles assemble at fatty acid-rich raftlike sites on the plasma membrane of the virus-producing cell. Several thousand copies of the Gag polyprotein colocalize at these sites and form spherical, budding particles. Freshly budded immature particles exhibit a doughnut-like appearance in electron micrographs, with Gag proteins located around the periphery of the particle under the plasma membrane-derived lipid bilayer (*1*). Soon after budding, Gag is processed by viral protease into the mature proteins of the infectious virion. These cleavage events are accompanied by profound morphological changes resulting in mature particles with a cone-shaped core at the center of the HIV-1 particle (*1, 2*).

The HIV-1 Gag polyprotein is comprised of several separate protein domains that include matrix (MA),[1] capsid (CA), nucleocapsid (NC), and p6 domains (*1*). In addition, there are two spacer peptides, p2 and p1, located between the CA and NC domains and between the NC and p6 domains, respectively. NMR and crystallographic efforts to determine the structure of full-length Gag have been unsuccessful, but structures of isolated protein domains have been determined.

The MA domain is important for targeting of Gag molecules to the plasma membrane. The N-terminal myristyl group and a conserved basic amino acid patch on the surface of the MA domain function synergistically to ensure tight membrane binding of Gag (*3*, *4*). High-resolution structural information is available for nonmyristylated and myristylated MA proteins (*5*−*7*). These studies indicated that entropic modulation rather than protein conformational changes regulates a myristyl switch in HIV-1 (*5*).

The CA domain appears to guide arrangements of Gag molecules during assembly. Small mutations within the CA domain were found to prevent particle assembly or interfere with correct assembly of the particle (*8*, *9*). Crystal structures of N- and C-terminal portions of the CA protein made it possible to localize a dimerization interface to the protein's C-terminal domain (*10*−*12*). Hydrogen−deuterium exchange experiments provided evidence for interactions between N- and C-terminal domains of CA specific to the mature virus (*13*, *14*). The importance of these regions for mature capsid assembly has been supported by mutational analysis (*15*, *16*).

The NC domain participates in the recognition and packaging of the viral RNA genome as well as binding to other cellular RNA molecules. The structures of the NC domain bound to cognate SL-2 or SL-3 sequences were determined by NMR (*17, 18*). These results revealed the flexible nature of NC, indicating that it can bind different nucleic acids in an adaptive manner. At the same time, RNA binding could promote Gag−Gag interactions by providing

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[1] Abbreviations: VLPs, viruslike particles; MA, matrix; CA, capsid; NC, nucleocapsid; $PI(4,5)P_2$, D-*myo*-phosphatidylinositol 4,5-diphosphate; IP5, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Q-TOF, quadrupole time-of-flight.

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a bridging platform for separate protein monomers (*19*). P6 does not appear to function in assembly per se but rather in the release of the assembled particle from the virus-producing cell (*20*−*22*).

Certain cellular factors that regulate Gag trafficking and assembly have been revealed (*23*, *24*). Ono et al. have recently suggested that phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] targets HIV-1 Gag to the plasma membrane (*25*). However, their studies did not distinguish between direct interactions of PI(4,5)P_2 with Gag and indirect effects mediated through other cellular factors. However, in vitro studies with a defined assembly system have shown that inositol phosphates (IP) and soluble analogues of phosphatidylinositol phosphates (PIP) modulate particle assembly (*26*) so that viruslike particles (VLPs) closely resembling authentic immature HIV-1 virion are formed from recombinant Gag in the presence of IP/PIP and nucleic acid (*26*). While both IP/PIP and nucleic acid have been shown to be essential for assembly of VLPs, how these cofactors interact with Gag and modulate assembly remains to be elucidated.

We present here mass spectrometric protein footprinting data for interactions of recombinant HIV-1 Gag with the assembly cofactors. Our results suggest that PI(4,5)P_2 binds to a cluster of basic amino acid residues in the MA domain while RNA could interact with lysines in the zinc fingers of the NC domain. Interestingly, our results suggest that specific protein−protein interactions involving the C-terminal region of the CA domain are promoted only when both PI(4,5)P_2 and RNA are included in the reaction mixture. The physiological relevance of our findings is discussed.

EXPERIMENTAL PROCEDURES

Recombinant HIV-1 Gag, Nucleic Acids, and Assembly of Particles. HIV-1 Gag protein was expressed in *Escherichia coli* and purified by a modification of a published technique (*27*). The recombinant protein lacked both the p6 domain and the N-terminal myristyl modification found on Gag protein produced in eukaryotic cells. D-*myo*-Phosphatidylinositol 4,5-diphosphate [PI(4,5)P_2] and D-*myo*-inositol 1,3,4,5,6-pentakisphosphate (IP5) were purchased from A.G. Scientific, Inc. (San Diego, CA). Yeast tRNA was purchased from Ambion, Inc. (Austin, TX). HPLC-purified ssDNA oligonucleotide (5′-AGGCTCCGCCCAGTGTGGAAAATC-TCTAGCA-3′) was obtained from Integrated DNA Technologies (Coralville, IA).

Protein Footprinting. Complexes of Gag with the assembly cofactors were preformed by mixing 20 μM Gag with the indicated concentrations of PI(4,5)P_2/IP5 and/or nucleic acid (tRNA or ssDNA) in a 12 μL reaction volume containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM DTT. The complexes were then subjected to modification with 400 μM *N*-hydroxysuccinimidobiotin (NHS-biotin) (Pierce, Rockford, IL). NHS-Biotin reacts specifically with primary amines on proteins, resulting in covalent addition of a biotin molecule (226.30 Da) to Lys residues with the concomitant release of *N*-hydroxysuccinimide. To obtain reproducible modification of surface lysines in free Gag and the preassembled Gag−PI(4,5)P_2−tRNA complexes, the Eppendorff tubes were placed on a shaker at 220 rpm and the biotinylation reactions were carried out at 25 °C for 30 min. The reactions were quenched with 10 mM (final concentration) lysine in its free amino acid form. The protein was denatured by adding 2% (w/v) SDS and incubating the mixture at 70 °C for 20 min. The Cys residues were then modified with 100 mM iodoacetamide at room temperature for 45 min. The reaction was quenched with 100 mM DTT, and the mixture was subjected to SDS−PAGE. Gag was visualized with Coomassie blue stain and excised. The gel slices were extensively destained in a 50% methanol/10% acetic acid mixture. SDS was removed via two 10 min washes with 50 mM NH_4HCO_3. The gel slices were then dehydrated with 100% acetonitrile, vacuum desiccated, and exposed to 1 μg of trypsin in 50 mM NH_4HCO_3 buffer at room temperature for 16 h. The supernatant was recovered and subjected to MS and MS/MS analysis.

Mass Spectrometric Analysis. MS analysis reveals the precise molecular weight of a peptide ion, while MS/MS analysis yields amino acid sequence information of the parent peptide ion based on internal fragmentation of peptide bonds. MS spectra were recorded using MALDI-TOF or Q-TOF techniques. MALDI-TOF experiments were performed with a Kratos Axima-CFR instrument using α-cyano-4-hydroxycinnamic acid as the matrix. MS and MS/MS analyses were carried out using a Micromass Q-TOF-II instrument equipped with an electrospray source and a Micromass cap liquid chromatograph. Peptides were separated with a Waters Symmetry 300 C18 precolumn and the Micro-Tech Scientific VC-10-C18-150 column using two sequential linear gradients from 5 to 40% and from 40 to 90% acetonitrile for 35 and 10 min, respectively. MS/MS analysis data and the Mascot search engine (http://www.matrixscience.com) were used to identify Gag peptide peaks from the NCBInr primary sequence database. The matched peptides were identified in the MS spectra. For accurate quantitative analysis of the biotinylated peptide peaks, the intensities of all MS peptide peaks during the entire run as well as at least two adjacent unmodified Gag peptide peaks were considered as controls.

Modeling Studies. The model for the MA−PI(4,5)P_2 complex was generated and minimized using SYBYL (version 6.8, Tripos Associates, St. Louis, MO). Polar hydrogens and electrostatic potentials were added to the available MA structure (*5*) by using SYBYL. Docking was performed with AUTODOC 3 (*28*). The grid was centered on the side chain of Lys32. One hundred dockings were calculated using the Lamarckian genetic algorithm (*28*). All other parameters remained at their default values.

RESULTS

The goal of our studies was to identify Gag contacts with the key assembly cofactors PI(4,5)P_2 and RNA. PI(4,5)P_2 is an integral part of the plasma membrane primarily exposed to the cytoplasmic surface. This lipid has been suggested to play a key role in targeting of Gag to the plasma membrane (*25*). In our studies, a commercially available, water-soluble PI(4,5)P_2 with shortened aliphatic tails was used. In parallel experiments, we also analyzed IP5, a soluble component of the cytoplasm which has been shown to contribute to the assembly of VLPs in vitro (*26*). Previous reports have conclusively shown that nucleic acid of virtually any sequence can support the assembly process (*27*, *29*). Therefore, we used either yeast tRNA or a 31-nucleotide ssDNA of an arbitrary sequence in the footprinting assays. The

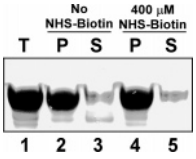

FIGURE 1: SDS−PAGE of assembled Gag−PI(4,5)P_2−tRNA complexes. Lane 1 contained the total Gag protein used in the reaction. Lanes 2 and 3 contained pellet and supernatant fractions, respectively, of the assembled Gag−PI(4,5)P_2−tRNA complex. Lanes 4 and 5 contained pellet and supernatant fractions, respectively, of the Gag−PI(4,5)P_2−tRNA complex that was first preassembled and then treated with 400 μM NHS-biotin. The reaction mixtures were centrifuged at 13 000 rpm for 1 h. Pellet and supernatant fractions were separately subjected to SDS−PAGE analysis, and Gag was visualized by Coomassie staining. Sedimentation allows the separation of the assembled VLPs (that readily pellet) from unassembled Gag (that remains in the solution). In both samples without (lanes 2 and 3) and with NHS-biotin treatment (lanes 4 and 5), the majority of the Gag protein was found in the pellet. T, P, and S indicate total, pellet, and supernatant fractions, respectively.

recombinant Gag protein used here forms spherical particles that closely resemble authentic immature virus particles in the presence of both nucleic acid and PI(4,5)P_2-related cofactors (*26*).

To map binding sites of these cofactors in Gag, our mass spectrometric footprinting method was employed (*30*−*32*). This approach exploits the differential accessibility of the primary amine modifying reagent NHS-biotin to lysine residues in the free protein versus the protein−ligand complex. The subsequent mass spectrometric analysis enables accurate identification of the lysine residues that are specifically protected by direct contacts with the cognate ligand (*30*−*32*). It seemed likely that binding sites on the protein for negatively charged ligands such as PI(4,5)P_2 would include lysines.

Prior to the footprinting of the Gag−PI(4,5)P_2−tRNA complex, it was important to confirm that the integrity of the assembled complex was preserved under our footprinting conditions. In previous studies, we have found that upon treatment of a HIV-1 reverse transcriptase−viral RNA−tRNA complex with 400 μM NHS-biotin, the enzyme retained greater than 97% of its activity (*30*). Similarly, the preassembled complex of human DNA replication protein A with ssDNA remained intact following the treatment with 400 μM NHS-biotin (*31*, *32*). We examined the Gag−PI(4,5)P_2−tRNA complex upon modification with 400 μM NHS-biotin. The Gag concentration was 20 μM, which provided the ratio of NHS-biotin to total Gag lysines of ∼1:1. The data in Figure 1 indicate that under such mild conditions no detectable dissociation of the preassembled complex was observed.

Our next goal was to obtain a detailed accessibility map of the free Gag protein by mass spectrometry. Assignment of tryptic fragments revealed greater than 90% amino acid sequence coverage of Gag (Figure 2). Biotinylated peptides were identified from MS spectra, and the modified Lys residues were revealed from MS/MS analysis. We observed modification of 21 lysines (Figure 2). While the structure of the full-length Gag protein is not available, all the modified lysines were found to be surface-exposed in the available structures of MA, CA, and NC domains (*5*−*7*, *10*−*12*, *17*).

To identify PI(4,5)P_2 binding site(s), we preformed the-Gag−PI(4,5)P_2 complex and subjected it to NHS-biotin modification. Of the 21 lysines readily modified in free Gag,

FIGURE 2: Summary of mass spectrometric analysis of free Gag. The protein was modified with 400 μM NHS-biotin and subsequently subjected to trypsin proteolysis. The peptide fragments detected by MALDI-TOF or Q-TOF are underlined. Gag sequences to which we assigned MS peptide peaks are highlighted in boldface type. The protein segments that could not be detected by MS are in lightface type. Modified lysines are denoted with the arrowed B.

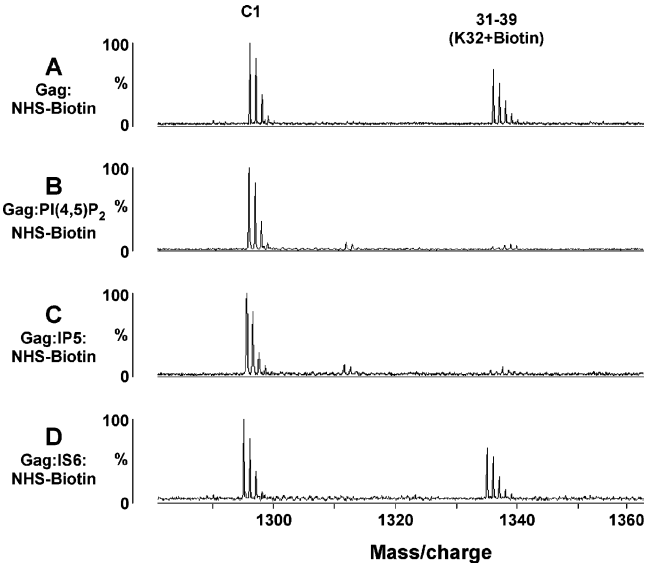

FIGURE 3: Segments of the MALDI-TOF spectra showing protection of K32 of Gag with PI(4,5)P_2 and IP5. (A) Free Gag was treated with 400 μM NHS-biotin. (B) PI(4,5)P_2 (100 μM) was added to 20 μM Gag to form the complex. The Gag−PI(4,5)P_2 structure was then exposed to NHS-biotin modification. The magnitude of the modified peptide peak was significantly diminished in the context of the complex, indicating protection of K32 from biotinylation by PI(4,5)P_2. (C) Addition of 100 μM IP5 also protected K32 from biotinylation. (D) The control indicates that 100 μM IS6 did not exhibit any protection of K32. Each Gag peptide peak resulted in a clearly resolved peak cluster, indicating monoisotopic resolution in our MS analysis. C1 is an unmodified tryptic peptide peak of Gag, which serves as an internal control.

only K30 and K32 were protected in the complex (Figure 3B and Table 1). IP5, which is structurally similar to PI(4,5)P_2, has been shown to support assembly of VLPs in vitro (*26*). In our footprinting experiments, IP5 also specifically protected K30 and K32 (Figure 3C). Inositol hexasulfate, which does not affect particle assembly (*26*), failed to protect lysines in Gag (Figure 3D).

Table 1: Biotinylation Patterns of Gag and Gag−PI(4,5)P_2, Gag−tRNA, and Gag−PI(4,5)P_2−tRNA Complexes[a]

	Gag with NHS-biotin	Gag−PI(4,5)P_2 with NHS-biotin	Gag−tRNA with NHS-biotin	Gag−PI(4,5)P_2−tRNA with NHS-biotin
K18	+	+	+	+
K26	+	+	+	+
K30	+	−	+/−	−
K32	+	−	+/−	−
K95	+	+	+	+
K103	+	+	+	+
K110	+	+	+	+
K114	+	+	+	+
K157	+	+	+	+
K272	+	+	+	+
K290	+	+	+	+
K302	+	+	+	+
K314	+	+	+	−
K335	+	+	+	+
K359	+	+	+	+
K391	+	+	−	−
K397	+	+	+	+
K410	+	+	+	+
K411	+	+	+	+
K415	+	+	+	+
K424	+	+	−	−

[a] A + indicates lysines readily modified by NHS-biotin. A +/− indicates lysines partly protected with tRNA (see the text for more details). A − indicates lysines fully protected in the Gag complexes with PI(4,5)P_2 or tRNA. Residues in boldface type are those where differences are observed in NHS-biotin susceptibility.

We observed partial protection of K30/K32 in the presence of 0.04 mg/mL (the concentration normally used in the assembly reactions) tRNA. These lysines could be fully protected with a 3-fold increase in tRNA concentration. We do not know how many Gag molecules bind to a tRNA molecule, but this number might be as high as 16 (*33*). We also employed short ssDNA to quantitatively compare the affinities of PI(4,5)P_2 and nucleic acid for Gag. Of note, short (31 bases) ssDNA can effectively support the assembly process in vitro (*26*, *27*, *29*). In parallel experiments, PI(4,5)P_2 and ssDNA were titrated and dose-dependent protections of K30/K32 were compared. Figure 4 indicates that 100 μM PI(4,5)P_2 fully prevented biotinylation of K32, while an at least 4-fold higher molar concentration of ssDNA was necessary to observe the protection. It should also be noted that each 31-nucleotide DNA molecule probably contains several binding sites.

Footprinting of the Gag−tRNA complex indicated protection of K391 and K424 located in the NC domain (Table 1). These protections were observed upon addition of 0.04 mg/mL tRNA (~1.5 μM) or 20 μM ssDNA to 20 μM Gag (data not shown). In contrast, the NC domain lysines remained fully susceptible to modification in the presence of 100 μM PI(4,5)P_2.

While tRNA supports assembly of very small VLPs, there is evidence suggesting that "correct" assembly of the HIV-1 particles requires both cofactors: PI(4,5)P_2 and nucleic acid (*26*). Therefore, our next goal was to footprint Gag in the presence of PI(4,5)P_2 and tRNA. Free Gag was fully soluble, while formation of the Gag−PI(4,5)P_2−tRNA complex yielded VLPs that could be pelleted by sedimentation (Figure 1). To achieve reproducible biotinylation of surface lysines in the free protein and the preassembled complex, the modification reactions were performed using a shaker at 220 rpm. The results indicated a similar extent of modification of the surface residues in the two samples.

We observed that K30 and K32, protected in the Gag−PI(4,5)P_2 complex, and K391 and K424, protected in the Gag−tRNA complex, were also protected in the context of the Gag−PI(4,5)P_2−tRNA complex (Table 1). Interestingly, we found that an additional lysine residue at position K314, located in the C-terminal part of the CA domain, was protected in the Gag−PI(4,5)P_2−tRNA complex (Figure 5). This protection was observed only when both PI(4,5)P_2 and tRNA were present in the reaction mixture (Figure 5).

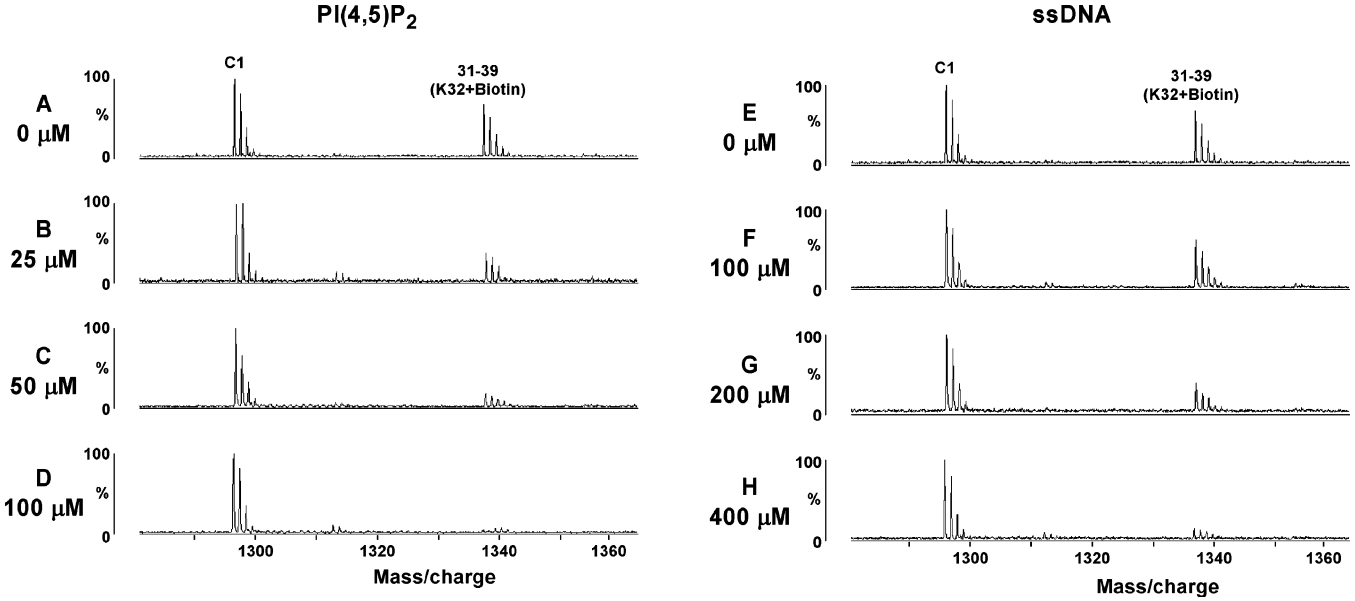

FIGURE 4: Relative affinities of PI(4,5)P_2 and ssDNA binding to recombinant HIV-1 Gag. Gag−PI(4,5)P_2 (left panel) and Gag−ssDNA (right panel) complexes were formed first and then treated with NHS-biotin. In all the experiments, the Gag concentration was 20 μM. The following concentrations of PI(4,5)P_2 or ssDNA were added to Gag: (A) no PI(4,5)P_2, (B) 25 μM PI(4,5)P_2, (C) 50 μM PI(4,5)P_2, (D) 100 μM PI(4,5)P_2, (E) no ssDNA, (F) 100 μM ssDNA, (G) 200 μM ssDNA, and (H) 400 μM ssDNA. The peptide peak of amino acids 31−39, containing modified K32, is designated. The data indicate that 100 μM PI(4,5)P_2 was sufficient to fully shield K32 from biotinylation, while at least 400 μM ssDNA was necessary to observe the protection.

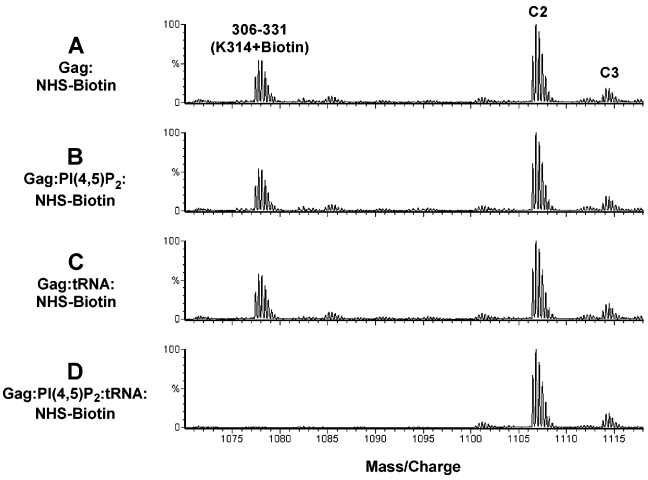

FIGURE 5: Segments of the MS spectra demonstrating specific protection of K314 in the context of the Gag−$PI(4,5)P_2$−tRNA complex. (A) Free Gag treated with NHS-biotin. (B) Gag−$PI(4,5)P_2$ complex modified with NHS-biotin. (C) Gag−tRNA complex treated with NHS-biotin. (D) Gag−$PI(4,5)P_2$−tRNA complex modified with NHS-biotin. Concentrations of Gag, $PI(4,5)P_2$, and tRNA were 20 μM, 100 μM, and 0.04 mg/mL (~1.5 μM), respectively. Control unmodified tryptic peptide peaks of Gag are denoted as C2 and C3.

In the experiments described above, $PI(4,5)P_2$ and IP5 yielded very similar patterns. Similarly, tRNA and ssDNA were interchangeable. For example, IP5 protected K30 and K32 of the MA domain, ssDNA interacted with K391 and K424 of the NC domain, and K314 was protected only when both IP5 and ssDNA were present in the reaction mixture. These findings are consistent with the previous observations that IP5 can serve as a cofactor in the in vitro assembly experiments and that both short ssDNA and cellular RNA could efficiently support the assembly process (*27, 29*).

DISCUSSION

We employed mass spectrometric footprinting to dissect interactions of HIV-1 Gag with the key assembly cofactors, $PI(4,5)P_2$ and RNA. Our results indicated that of 21 surface lysines readily susceptible to biotinylation in the free polyprotein, only K30 and K32 in the MA domain were strongly protected from modification by $PI(4,5)P_2$. In contrast, nucleic acid protected K391 and K424 in the NC domain. Intriguingly, the concerted binding of both $PI(4,5)P_2$ and nucleic acid also resulted in protection of K314 in the CA domain.

While there is structural information from crystallographic and NMR studies on isolated domains of Gag (*5*−*7*, *10*−*12*, *17*), there is no direct structural information about these regions of the full-length Gag protein. One important result emerging from our studies is that the lysine residues exposed to solvent in the isolated domains are also exposed in Gag protein. This finding lends support for the use of the existing structural data (*5*−*7*, *10*−*12*, *17*) in studies of Gag structure.

Our previous studies on footprinting of related systems indicated excellent agreement between the mass spectrometric results and available crystal structure data on corresponding nucleoprotein complexes (*30*−*32*). At the same time, it was revealed that protections may arise not only from direct ligand−protein interactions but also from ligand-induced protein−protein contacts (*30*−*32*). Therefore, to accurately interpret the protection profiles observed in the Gag−$PI(4,5)P_2$−tRNA complex, we considered available biological and structural data (see below).

Site-directed mutagenesis studies revealed that a cluster of basic residues located in the MA domain, together with N-terminal myristylation, plays a critical role in tight binding of Gag to the plasma membrane (*4*, *34*). The K26/K27 and K30/K32 double substitutions significantly inhibited total virion production, while alterations of K18, R20, and R22 had no measurable effects (*34*). Consistent with the site-directed mutagenesis data, our results indicate that K30 and K32 were protected by $PI(4,5)P_2$, while K18 remained fully surface exposed in the Gag−$PI(4,5)P_2$ complex (Table 1). The surface accessibility of R20 and R22 could not be determined using lysine-specific NHS-biotin. In our footprinting experiments, we could not observe peptides containing biotinylated K27 due probably to very short fragments generated by trypsin hydrolysis. K26 was surface exposed and readily modified in free Gag or in the Gag−$PI(4,5)P_2$ complex (Table 1).

Using the existing structural data on free MA protein, we used molecular modeling to examine whether K30 and K32 could be a plausible binding site for $PI(4,5)P_2$. Our model suggests that K27, K30, and K32 could contribute to the direct binding to $PI(4,5)P_2$ (Figure 6A). This notion is further strengthened by previous findings that the Gag mutant lacking residues 16−99 of the MA protein formed VLPs in vitro in the presence of nucleic acid alone, without addition of $PI(4,5)P_2$ or IP5 (*26*, *29*). Our modeling studies suggested that in addition to the basic patch between residues 27 and 32, residues Q28 and H33 could also contribute to $PI(4,5)P_2$ binding (Figure 6B). The latter residue is highly conserved among different HIV-1, HIV-2, and SIV isolates. Glutamine or lysine is found at position 28 in different HIV-1 strains. Fortunately, two different NMR structures of the MA proteins, one with K28 (*7*) and another with Q28 (*5*, *6*), are available. Comparison of these two structures in our modeling studies indicated that the amines of either K28 or Q28 could form hydrogen bonds with $PI(4,5)P_2$. In any case, our results (Figure 3) together with previous site-directed mutagenesis studies (*4*, *34*) and the results of molecular modeling (Figure 6) are all consistent with the idea that the MA domain of Gag can interact directly with $PI(4,5)P_2$.

$PI(4,5)P_2$ and IP5 exhibited very similar patterns in our mass spectrometric footprinting. These two compounds are structurally very similar, and IP5 has been shown to support assembly of VLPs in vitro. However, HIV-1 particles in vivo normally assemble at the plasma membrane. $PI(4,5)P_2$ is an integral part of the plasma membrane, while IP5 is a soluble component of the cytoplasm. The depletion of cellular $PI(4,5)P_2$ by overexpression of phosphoinositide 5-phosphatase IV resulted in severe reduction of the virus production (*25*). Furthermore, in cells expressing Arf6/Q67L, which induces the formation of $PI(4,5)P_2$-enriched endosomal structure, Gag was redirected to the $PI(4,5)P_2$-enriched vesicles and HIV-1 virions budded into these vesicles (*25*). On the basis of these findings, Ono et al. suggested that $PI(4,5)P_2$ could promote or stabilize binding of Gag to the plasma membrane by interacting with Gag directly, or indirectly through an unknown adaptor molecule. The work presented here suggests that $PI(4,5)P_2$ could directly bind Gag by interacting with the basic pocket of the MA domain.

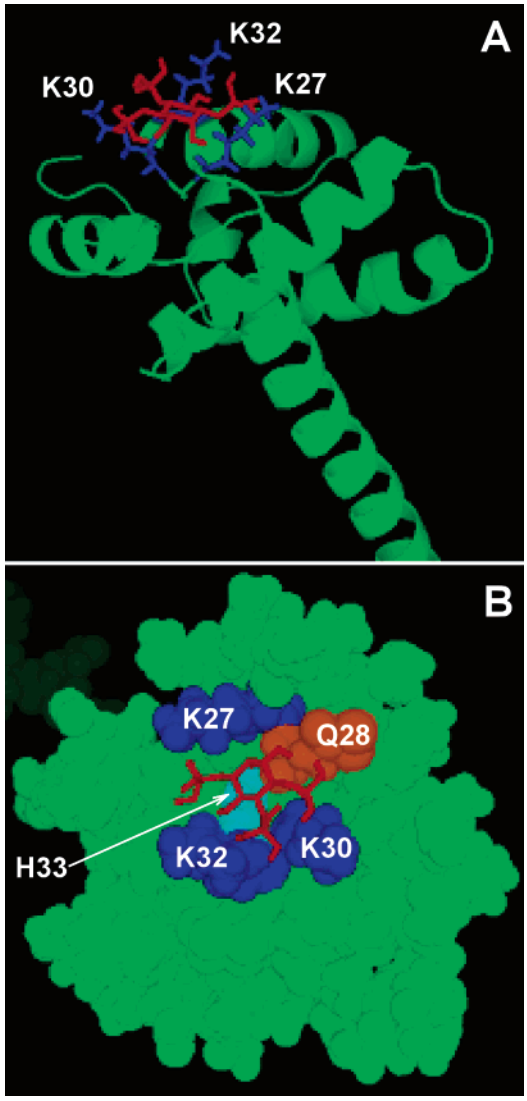

FIGURE 6: Model for the MA−PI(4,5)P_2 complex generated and minimized using SYBYL (version 6.8, Tripos Associates, St. Louis, MO). Polar hydrogens and electrostatic potentials were added to the MA structure by using SYBYL. Docking was performed with AUTODOC 3. The grid was centered on the side chain of Lys32. One hundred dockings were calculated using the Lamarckian genetic algorithm. All other parameters remained at their default values. (A) "Side view" of the complex in which the basic residues (K27, K30, and K32) that could directly interact with PI(4,5)P_2 are colored blue and PI(4,5)P_2 is colored red. (B) "Top view" of the complex which reveals that the imidazole group of H33 (cyan) and Q28 (orange) could hydrogen bond with PI(4,5)P_2 (red).

We suggest that these interactions could play a key role in targeting of Gag to the plasma membrane, as well as in modulating particle assembly.

Our mass spectrometric footprinting indicated that both PI(4,5)P_2 and nucleic acid could bind K30 and K32. Obviously, electrostatic interactions contribute to these interactions. However, these cannot explain all our observations. For example, we found that the strong anionic compound inositol hexasulfate (IS6) did not provide any detectable protection of K30 or K32 (Figure 3D). When interactions of Gag with tRNA are being analyzed, it is important to consider that such a large nucleic acid contains multiple Gag binding sites (*33*). Therefore, we used short ssDNA to quantitatively compare the affinities of PI(4,5)P_2 and nucleic acid for Gag. The data in Figure 4 show that PI(4,5)P_2 has a higher affinity with respect to K30 and K32 than short ssDNA. Taken together, the available evidence is consistent with the hypothesis that PI(4,5)P_2 is the preferred ligand for the basic patch of the MA domain.

The NC domain of Gag is highly basic. We found that K391 and K424, which are located in the two zinc fingers of the NC domain, were protected in the Gag−tRNA complex. These two lysine residues make direct contact with RNA in the NC−SL3 RNA complex, as shown by NMR (*17*). In the NC−SL2 RNA complex, in which NC assumes a different structure (*18*), K411 forms a salt bridge with a phosphodiester group in the RNA backbone (*18*). We found that K397, K410, K411, and K415 remain solvent exposed in the Gag−tRNA complex, as they are in the NC−SL3 complex (*17*). In conclusion, the results show that the NC domain of Gag can bind tightly to nucleic acid, but not to PI(4,5)P_2. Thus, the data suggest that the NC domain, while it is flexible enough to accommodate different nucleic acid structures, does not provide an appropriate cluster, such as that found in the MA domain, to enable efficient binding to PI(4,5)P_2.

Since virtually any nucleic acid can support assembly in vitro (*27*, *29*), and since efficient assembly in vivo is RNA-dependent but does not require viral RNA (*35*−*37*), we used yeast tRNA and a small oligodeoxynucleotide in these studies. Our results which show that both tRNA and ssDNA could bind tightly to the NC domain of Gag are consistent with the previous studies which show that oligonucleotides of arbitrary sequence can effectively support the assembly process (*27*, *29*).

We observed that K30 and K32 of the MA domain were efficiently protected in the Gag−PI(4,5)P_2−tRNA ternary complex (Table 1). Similarly, K391 and K424 were protected in the ternary complex. Since the former two residues were efficiently protected by PI(4,5)P_2 and the latter two were protected only by nucleic acids in binary complexes, we infer that PI(4,5)P_2 protects K30 and K32 and tRNA protects K391 and K424 in the ternary complex.

Remarkably, we observed protection of K314 within the CA domain only when both PI(4,5)P_2 and tRNA were present in the reaction mixture (Figure 5). The previous reports indicated that both PIP/IP and nucleic acid are essential for assembly of VLPs (*26*). Crystallographic and site-directed mutagenesis studies revealed that the α-helical region spanning amino acids 178−192 of the CA protein (corresponding to amino acids 311−324 of Gag, which includes K314) is critical for stabilizing the C-terminal dimer of CA (*11*). Hydrogen−deuterium exchange experiments also implicated this protein segment in CA−CA interactions (*13*). Therefore, we suggest that the observed protection of K314 is due to protein−protein interactions between Gag molecules. Importantly, our data indicate that these interactions can only occur when both cofactors, i.e., PI(4,5)P_2 and RNA, bind the Gag polyprotein. Thus, the protection of K314 appears to correlate with the altered Gag−Gag interactions leading to assembly of VLPs (*26*).

In summary, our mass spectrometric protein footprinting yielded new and important information about Gag−PI(4,5)P_2−nucleic acid interactions. At the same time, the observation that K314 is specifically protected in the biologically relevant Gag−PI(4,5)P_2−RNA structure opens a doorway to the development of a novel powerful method

for initial screening of anti-assembly inhibitors. Of note, such a method can be adequately rapid and require minute amounts of the protein and cofactors.

REFERENCES

1. Swanstrom, R., and Wills, J. W. (1997) Synthesis, assembly, and processing of viral proteins, in *Retroviruses* (Coffin, J. M., Hughes, S. H., and Varmus, H. E., Eds.) pp 263−334, Cold Spring Harbor Laboratory Press, Woodbury, NY.
2. Li, S., Hill, C. P., Sundquist, W. I., and Finch, J. T. (2000) Image reconstructions of helical assemblies of the HIV-1 CA protein, *Nature 407* (6802), 409−13.
3. Yuan, X., Yu, X., Lee, T. H., and Essex, M. (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* (11), 6387−94.
4. Zhou, W., Parent, L. J., Wills, J. W., and Resh, M. D. (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* (4), 2556−69.
5. Tang, C., Loeliger, E., Luncsford, P., Kinde, I., Beckett, D., and Summers, M. F. (2004) Entropic switch regulates myristate exposure in the HIV-1 matrix protein, *Proc. Natl. Acad. Sci. U.S.A. 101* (2), 517−22.
6. Hill, C. P., Worthylake, D., Bancroft, D. P., Christensen, A. M., and Sundquist, W. I. (1996) Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: Implications for membrane association and assembly, *Proc. Natl. Acad. Sci. U.S.A. 93* (7), 3099−104.
7. Matthews, S., Barlow, P., Clark, N., Kingsman, S., Kingsman, A., and Campbell, I. (1995) Refined solution structure of p17, the HIV matrix protein, *Biochem. Soc. Trans. 23* (4), 725−9.
8. Dorfman, T., Bukovsky, A., Ohagen, A., Hoglund, S., and Gottlinger, H. G. (1994) Functional domains of the capsid protein of human immunodeficiency virus type 1, *J. Virol. 68* (12), 8180−7.
9. Chazal, N., Carriere, C., Gay, B., and Boulanger, P. (1994) Phenotypic characterization of insertion mutants of the human immunodeficiency virus type 1 Gag precursor expressed in recombinant baculovirus-infected cells, *J. Virol. 68* (1), 111−22.
10. Worthylake, D. K., Wang, H., Yoo, S., Sundquist, W. I., and Hill, C. P. (1999) Structures of the HIV-1 capsid protein dimerization domain at 2.6 Å resolution, *Acta Crystallogr. D55* (Part 1), 85−92.
11. Gamble, T. R., Yoo, S., Vajdos, F. F., von Schwedler, U. K., Worthylake, D. K., Wang, H., McCutcheon, J. P., Sundquist, W. I., and Hill, C. P. (1997) Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein, *Science 278* (5339), 849−53.
12. Momany, C., Kovari, L. C., Prongay, A. J., Keller, W., Gitti, R. K., Lee, B. M., Gorbalenya, A. E., Tong, L., McClure, J., Ehrlich, L. S., Summers, M. F., Carter, C., and Rossmann, M. G. (1996) Crystal structure of dimeric HIV-1 capsid protein, *Nat. Struct. Biol. 3* (9), 763−70.
13. Lanman, J., Lam, T. T., Barnes, S., Sakalian, M., Emmett, M. R., Marshall, A. G., and Prevelige, P. E., Jr. (2003) Identification of novel interactions in HIV-1 capsid protein assembly by high-resolution mass spectrometry, *J. Mol. Biol. 325* (4), 759−72.
14. Lanman, J., Lam, T. T., Emmett, M. R., Marshall, A. G., Sakalian, M., and Prevelige, P. E., Jr. (2004) Key interactions in HIV-1 maturation identified by hydrogen−deuterium exchange, *Nat. Struct. Mol. Biol. 11* (7), 676−7.
15. von Schwedler, U. K., Stray, K. M., Garrus, J. E., and Sundquist, W. I. (2003) Functional surfaces of the human immunodeficiency virus type 1 capsid protein, *J. Virol. 77* (9), 5439−50.
16. Ganser-Pornillos, B. K., von Schwedler, U. K., Stray, K. M., Aiken, C., and Sundquist, W. I. (2004) Assembly properties of the human immunodeficiency virus type 1 CA protein, *J. Virol. 78* (5), 2545−52.
17. De Guzman, R. N., Wu, Z. R., Stalling, C. C., Pappalardo, L., Borer, P. N., and Summers, M. F. (1998) Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element, *Science 279* (5349), 384−8.
18. Amarasinghe, G. K., De Guzman, R. N., Turner, R. B., and Summers, M. F. (2000) NMR structure of stem-loop SL2 of the HIV-1 psi RNA packaging signal reveals a novel A-U-A base-triple platform, *J. Mol. Biol. 299* (1), 145−56.
19. Bennett, R. P., Nelle, T. D., and Wills, J. W. (1993) Functional chimeras of the Rous sarcoma virus and human immunodeficiency virus gag proteins, *J. Virol. 67* (11), 6487−98.
20. Freed, E. O. (2002) Viral late domains, *J. Virol. 76* (10), 4679−87.
21. Pornillos, O., Garrus, J. E., and Sundquist, W. I. (2002) Mechanisms of enveloped RNA virus budding, *Trends Cell Biol. 12* (12), 569−79.
22. Gottlinger, H. G., Dorfman, T., Sodroski, J. G., and Haseltine, W. A. (1991) Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release, *Proc. Natl. Acad. Sci. U.S.A. 88* (8), 3195−9.
23. Stuchell, M. D., Garrus, J. E., Muller, B., Stray, K. M., Ghaffarian, S., McKinnon, R., Krausslich, H. G., Morham, S. G., and Sundquist, W. I. (2004) The human endosomal sorting complex required for transport (ESCRT-I) and its role in HIV-1 budding, *J. Biol. Chem. 279* (34), 36059−71.
24. Dong, X., Li, H., Derdowski, A., Ding, L., Burnett, A., Chen, X., Peters, T. R., Dermody, T. S., Woodruff, E., Wang, J. J., and Spearman, P. (2005) AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly, *Cell 120* (5), 663−74.
25. Ono, A., Ablan, S. D., Lockett, S. J., Nagashima, K., and Freed, E. O. (2004) Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane, *Proc. Natl. Acad. Sci. U.S.A. 101* (41), 14889−94.
26. Campbell, S., Fisher, R. J., Towler, E. M., Fox, S., Issaq, H. J., Wolfe, T., Phillips, L. R., and Rein, A. (2001) Modulation of HIV-like particle assembly in vitro by inositol phosphates, *Proc. Natl. Acad. Sci. U.S.A. 98* (19), 10875−9.
27. Campbell, S., and Rein, A. (1999) In vitro assembly properties of human immunodeficiency virus type 1 Gag protein lacking the p6 domain, *J. Virol. 73* (3), 2270−9.
28. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) Automated Docking Using a Lamarckian Genetic Algorithm and an Empirical Binding Free Energy Function, *J. Comput. Chem. 19* (14), 1639−62.
29. Gross, I., Hohenberg, H., Wilk, T., Wiegers, K., Grattinger, M., Muller, B., Fuller, S., and Krausslich, H. G. (2000) A conformational switch controlling HIV-1 morphogenesis, *EMBO J. 19* (1), 103−13.
30. Kvaratskhelia, M., Miller, J. T., Budihas, S. R., Pannell, L. K., and Le Grice, S. F. (2002) Identification of specific HIV-1 reverse transcriptase contacts to the viral RNA:tRNA complex by mass spectrometry and a primary amine selective reagent, *Proc. Natl. Acad. Sci. U.S.A. 99* (25), 15988−93.
31. Liu, Y., Kvaratskhelia, M., Hess, S., Qu, Y., and Zou, Y. (2005) Modulation of replication protein A function by its hyperphosphorylation-induced conformational change involving DNA binding domain B, *J. Biol. Chem.* (in press).
32. Shell, S. M., Hess, S., Kvaratskhelia, M., and Zou, Y. (2005) Mass spectrometric identification of lysines involved in the interaction of human replication protein a with single-stranded DNA, *Biochemistry 44* (3), 971−8.
33. Fisher, R. J., Rein, A., Fivash, M., Urbaneja, M. A., Casas-Finet, J. R., Medaglia, M., and Henderson, L. E. (1998) Sequence-specific binding of human immunodeficiency virus type 1 nucleocapsid protein to short oligonucleotides, *J. Virol. 72* (3), 1902−9.
34. Freed, E. O., Englund, G., and Martin, M. A. (1995) Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection, *J. Virol. 69* (6), 3949−54.
35. Levin, J. G., Grimley, P. M., Ramseur, J. M., and Berezesky, I. K. (1974) Deficiency of 60 to 70S RNA in murine leukemia virus particles assembled in cells treated with actinomycin D, *J. Virol. 14* (1), 152−61.
36. Berkowitz, R., Fisher, J., and Goff, S. P. (1996) RNA packaging, *Curr. Top. Microbiol. Immunol. 214*, 177−218.
37. Muriaux, D., Mirro, J., Harvin, D., and Rein, A. (2001) RNA is a structural element in retrovirus particles, *Proc. Natl. Acad. Sci. U.S.A. 98* (9), 5246−51.

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