Retroviruses Human Immunodeficiency Virus and Murine Leukemia Virus Are Enriched in Phosphoinositides

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Retroviruses acquire a lipid envelope during budding from the membrane of their hosts. Therefore, the composition of this envelope can provide important information about the budding process and its location. Here, we present mass spectrometry analysis of the lipid content of human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MLV). The results of this comprehensive survey found that the overall lipid content of these viruses mostly matched that of the plasma membrane, which was considerably different from the total lipid content of the cells. However, several lipids are enriched in comparison to the composition of the plasma membrane: (i) cholesterol, ceramide, and GM3; and (ii) phosphoinositides, phosphorylated derivatives of phosphatidylinositol. Interestingly, microvesicles, which are similar in size to viruses and are also released from the cell periphery, lack phosphoinositides, suggesting a different budding mechanism/location for these particles than for retroviruses. One phosphoinositide, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], has been implicated in membrane binding by HIV Gag. Consistent with this observation, we found that PI(4,5)P₂ was enriched in HIV-1 and that depleting this molecule in cells reduced HIV-1 budding. Analysis of mutant virions mapped the enrichment of PI(4,5)P₂ to the matrix domain of HIV Gag. Overall, these results suggest that HIV-1 and other retroviruses bud from cholesterol-rich regions of the plasma membrane and exploit matrix/PI(4,5)P₂ interactions for particle release from cells.

Retroviruses rely on their host for many essential parts of the viral replication cycle. Biochemical and antibody-based analyses of the replication cycle and proteins found in the virions have revealed many details of the molecular interactions between human immunodeficiency virus (HIV) and its host (20). In contrast, the role of lipids has been less well studied. With the increasing recognition that lipids play an important role in cellular signaling, it is no coincidence that lipid factors are slowly gaining prominence in our understanding of retroviral replication.

Retroviruses, including HIV and murine leukemia virus (MLV), acquire their lipid coats by budding through host plasma membranes. Two important issues arise when considering the roles of lipids in retrovirus assembly and budding. First, the idea that HIV and other retroviruses bud from lipid rafts has gained widespread acceptance (39, 45). Lipid rafts are liquid ordered domains that exist within the liquid disordered phase of the bulk cell membrane. These dynamic lipid-protein assemblies are characterized by high levels of cholesterol, sphingolipids, saturated glycerophospholipids, and raft proteins. Because the half-lives for lipid rafts are extremely short (50), the assignment of HIV to lipid rafts is commonly established through the colocalization of HIV proteins with putative raft proteins and the preponderance of raft lipids, including cholesterol, sphingomyelin (SM), dihydrosphingomyelin (dHSM), ceramide (Cer), and glucosylceramide (Gluc-Cer) (8, 36).

The second issue is the role of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] in retrovirus assembly. Although PI(4,5)P₂ comprises only a small fraction of the total phospholipids in a typical animal cell, it plays multiple roles in regulating many cell signaling pathways. One such function is in the targeting of proteins with polybasic amino acid clusters to the plasma membrane (23, 60). Gag proteins, whose matrix domain (MA) contains a polybasic cluster in the globular domain, rely on a similar targeting mechanism for its accumulation at the plasma membrane prior to budding (10, 35). Indeed, the depletion of PI(4,5)P₂ inhibits virus assembly and leads to an accumulation of Gag at late endosomes and multivesicular bodies (38). Further, it has been argued that PI(4,5)P₂ acts both as a trigger for myristate exposure and membrane anchor (49).

The composition of the retroviral lipid envelope provides important information about the assembly and budding process, especially regarding the nature of the budding site. Early work in this field examined the lipid composition of Rous sarcoma virus (42–44) and HIV (1) but at low resolution using thin-layer chromatography. A more recent study by Brugger et al. used electrospray ionization mass spectrometry (ESI-MS) to compare the lipid composition of HIV with that of total cell membrane and suggested that HIV buds from a unique or specialized type of lipid raft (8). To better determine what
lipids are enriched or depleted in virions during budding, we analyzed the total lipid composition from highly purified samples of HIV and MLV by MS and compared it to that of the membrane from which these viruses bud, the plasma membrane. In addition, we have expanded the scope of the previous analyses to include more lipid classes and quantify the relative levels of glycerophosphatidylinositol (PI), glycerophosphatidylinositol monophosphate (PIP), glycerophosphatidylinositol bisphosphate (PIP2), other glycerophosphatidylethanolamine (ePC), and glycosphingolipid GM3. We find that retroviral envelopes resemble the lipid composition of plasma membrane except that they are highly enriched in specific raft lipids and phosphoinositides PIP and PIP2. In addition, we demonstrate that the source of PIP2 enrichment in the HIV envelope maps to the globular head of the MA domain of HIV Gag which contains the polybasic cluster, suggesting that the electrostatic interaction between MA and PIP2 is a conserved function in proper retroviral assembly and budding.

**MATERIALS AND METHODS**

Reagents. All cell culture medium and supplements were purchased from Gibco, Invitrogen (Carlsbad, CA). Lipid standards were purchased from Avanti Polar Lipid Inc. (Alabaster, AL) and Echelon Biosciences Inc. (Salt Lake City, UT). All other reagents, including high-performance liquid chromatography (HPLC)-grade methanol, chloroform, and pipedine, were purchased from Sigma Aldrich (St. Louis, MO).

Cell lines. Uninfected H9 cells and the chronically HIV type 1 HIV-1NL4-3-infected cell line, clone 4 (41), were cultured in RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin G at 100 U/ml, and streptomycin sulfate at 100 μg/ml (complete medium). Uninfected and chronically MLV-infected rat embryo fibroblast (REF) cell lines were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin G at 100 U/ml, and streptomycin sulfate at 100 μg/ml (complete medium).

Isolation and culture of macrophages. Elutriated monocytes from HIV-negative donor leukopacs were obtained from the NIH Transfusion Branch and cultured at 2 × 10^6 cells to 3 × 10^6 cells per well on ultra-low-attachment six-well Costar plates (catalog no. 3471; Corning, Acton, MA) in RPMI 1640 medium with the supplements described above (complete medium) for 7 days to generate monocyte-derived macrophages (MDMs). MDMs were infected with the CCR5-tropic NL4-3 (18) overnight followed by two washes with phosphate-buffered saline to remove nonadhered virus. The infected cells were then cultured, and supernatants were periodically removed for virus isolation.

Concentration of MLV. Concentrated MLV was produced in roller culture bottles from chronically infected REFs. The culture supernatants were passed through a 0.45-μm filter and ultracentrifuged at 25,000 rpm at 4°C for 90 min through a 15% sucrose layer to obtain purified virus. For HIV produced from clone 4 and MDMs, the culture supernatants were passed through a 0.45-μm filter and sedimented through 15% sucrose cushion, and the resulting HIV pellet was further purified away from contaminating microvesicles using anti-CD45 magnetic depletion (55). All virus and microvesicle (prepared from uninfected cells) stocks were stored at −80°C until use.

Preparation of VLP. Constructs expressing wild-type and ΔMA HIV-Gag in the absence ofEnv were made based on pNL4-3/KFS (a gift from Eric Freed, NCI, Frederick, MD). Pol was deleted, and a hemagglutinin tag was added to the C terminus of Gag to study the release of virus-like particles (VLP) in the absence of protease. To make a ΔMA HIV-Gag expression vector, the globular head (amino acids 8 to 126) was deleted from the modified NL4-3/KFS clone. HEK293 cells were transfected with wild-type and ΔMA HIV expression vector. At 48 h posttransfection, the supernatants were collected and passed through a 0.45-μm filter. The clarified supernatants were ultracentrifuged at 25,000 rpm at 4°C for 3 h through a 15% sucrose layer to obtain purified virus.

Plasma membrane extraction from cells. Plasma membrane fractions were purified using cationic colloidal silica beads (a gift from Donna Beer Stolz, McGowan Institute for Regenerative Medicine, University of Pittsburgh) at 1% (wt/vol) and then to polyacrylic acid with a molecular weight of 100,000; Aldrich, St. Louis, MO). The bound cells were then incubated in a hypotonic lysis buffer for 30 min before being lysed using a cell homogenizer followed by centrifugation at 900 × g to remove released internal membranes. The plasma membranes in the resulting pellet were purified away from the nuclei by sedimentation through a 70% Histodenz (Aldrich) cushion using a SW41 rotor at 20,000 rpm for 30 min. The purified plasma membrane pellet was then washed thoroughly with the lysis buffer to remove any residual Histodenz.

**Lipid preparation.** Total lipid samples were prepared using a modified version of the method of Bligh and Dyer (4). All buffers and reagents were prechilled in an ice bath. Virus or cells were washed and resuspended in 50 μl phosphate-buffered saline. A portion (0.6 ml) of a chloroform-methanol (1:2) mixture was added to the sample, and the mixture was vortexed vigorously three times for 1 min each time with a 5-min interval between the vortexing steps. Next, 0.3 ml chloroform and 0.2 ml of 1 M KCl was added to the tube, and the mixture was again vortexed, three times for 30 s each time with 1-min intervals between the vortexing steps. The mixture was then centrifuged for 2 min at 9,000 rpm to separate the phases. The lower organic layer was transferred to a clean microcentrifuge tube and dried under a stream of N2 gas or via a speed vacuum. Phosphoinositide-enriched lipid samples were prepared by replacing the 1 M KCl solution with 1 M HCl (58). Sphingolipid samples were prepared via the alkaline hydrolysis method described by Merrill (32). Briefly, 0.75 ml of chloroform and 0.3 ml of water were added to the tube, and the mixture was then centrifuged for 30 min at 80°C. After cooling, all the organic phases were discarded, and 1 ml of a 50 mM KCl solution was added to the sample, and the mixture was sonicated for 1 min, then centrifuged at 18,000 rpm for 1 h. The lower aqueous layer was removed through a 0.45-μm filter. The clarified supernatants were ultracentrifuged at 25,000 rpm at 4°C for 3 h through a 15% sucrose layer to obtain purified virus.

At 48 h posttransfection, the supernatants were collected and passed through a 0.45-μm filter and ultracentrifuged at 25,000 rpm at 4°C for 90 min through a 15% sucrose layer to obtain purified virus.

Analysis of lipids using ESI-MS. Qualitative lipid profiling via ESI-MS was carried out with a Waters Micromass Q-TOF micromass spectrometer with an up-front Waters CapLC inlet (Waters Corp., Milford, MA) as described previously (52). The capillary voltage and sample cone voltage were maintained at 3.0 kV and 70 V, respectively. The source temperature was set at 120°C, and the desolvation temperature was set at 250°C. Mass spectra were acquired in the negative ion mode with an acquisition time of 3 min, using chloroform-methanol (1:1, vol/vol) at a flow rate of 15 μl/min as the mobile phase. Typically, samples were dissolved in the mobile phase to give an appropriate concentration, and 2 μl of sample was injected for analysis.

For quantitative analysis, we used a triple quadrupole instrument ABI 4000QQT (Applied Biosystems, Foster City, CA) in the multiple reaction monitoring mode. This method is optimized to detect only specified ions of interest, thus increasing sensitivity and selectivity of detection (14, 32). In our experiments, the internal standards used included 1,2-dimyristoyl-glycerol-phosphocholine, 1,2-dimyristoyl-glycerol-3-phosphoethanolamine, 1,2-dimyristoyl-glycerol-3-phosphocholine, lauroyl sphingomyelin, N-heptadecanoyl-1-erythro-sphingosine (C17 ceramide), and N-myristoyl-1-N-octanoyl-1-erythro-sphingosine (C18 ceramide) (Polar Lipids), which allowed the measurement of glycerophosphatidylserine (PS), glycerophosphatidylethanolamine (PE) and phospholipase-PE (psPE), glycerophosphatidylcholine (PC) and ePC, SM, and Cer and Glu-Cer, respectively (see Fig. S5A in the supplemental material). PIP, PIP2, and PIP3 levels were referenced to 1,2-diacyl-glycerol-3-phosphoinositol, 1,2-diacyl-glycerol-3-phosphoinositol-4-phosphate, and 1,2-diacyl-glycerol-3-phosphoinositol-4,5-bisphosphate (Echelon Biosciences, Inc.), respectively (see Fig. S5B in the supplemental material). Since a suitable standard was not available for GM3, we normalized GM3 levels to SM levels. The total lipid and sphingolipid extracts were dissolved in chloroform-methanol (1:1, vol/vol), and typically, 10 to 15 μl of the sample was injected via an autosampler. For the phosphoinositide samples, the lipids were dissolved in chloroform-methanol (1:1), spiked with 1/10 volume of 300 ppm pipidine solution, and directly infused into the mass spectrometer (58). The m/z transitions used were published previously (7, 32, 58), and we optimized the declustering potential and collision energy using the Quantitative Optimization function available for the Analyst 1.4.1 software. The instrument was calibrated using polypropylene glycol standards provided by the manufacturer (Applied Biosystems), and mass tolerance was adjusted to ±100 ppm or 0.1 Da. The signal intensity obtained for each lipid species was converted to their level (moles) in each fraction by normalization to the appropriate internal standard. The final lipid molar percentage (the amount of each lipid was measured in moles and then converted to a percentage of the total) of each sample was obtained by cross normalizing the lipid level of each fraction measured (25). The standard deviations shown represent the differences in at least three replicate experiments, i.e., n ≥ 3.
For the measurement of cholesterol, we employed the same ABI 4000QTR instrument connected to an HPLC system using a sensitive HPLC-ESI-MS method (G. Shiui et al., unpublished data). Briefly, lipids were separated on an Agilent Zorbax Eclipse XDB-C18 column (inner diameter, 4.6 mm; length, 150 mm) (Agilent Technologies, Santa Clara, CA) at 30°C using chloroform–methanol–0.1 M ammonium acetate (100:100:4 [vol/vol/vol]) as a mobile phase at a flow rate of 0.4 ml min⁻¹ and an injection volume of 30 μl. MS was recorded at both positive and negative ESI modes in enhanced MS scan mode with a Turbo spray source voltage of +5.000 V and −4.500 V, respectively, and a source temperature of 250°C. A total run time of 30 min was utilized to elute both polar lipids and neutral lipids. The cholesterol-to-PC molar ratio (cholesterol/PC ratio) was used to compare the cholesterol levels in the different samples (8).

The cholesterol levels were calculated by normalizing the 369 m/z peak intensity to the total PC peak intensity, both of which were measured at positive mode.

Virus budding and release assays. To assess HIV release, 2.5 × 10⁵ HEK293 cells in 24 wells were transfected with 150 ng of replication-competent HIV pNL4-3 construct (NIH AIDS Reagent Program) together with plasmids expressing 5-phosphatase IV or catalytically inactive 5-phosphatase ΔI (gifts from Eric Freed, NCI, Frederick with permission from P. Majerus, Washington University School of Medicine, St. Louis, MO) or with an empty vector control. For MLV release assays, 200 ng of plasmid MLV Env-GFP encoding full-length Friend 57 MLV genome with a green fluorescent protein (GFP) insertion into the envelope protein (28, 51) was cotransfected together with plasmids encoding 5-phosphatase IV or catalytically inactive 5-phosphatase ΔI or with a vector control as described above. At 48 h posttransfection, the released virus infectivity was measured by titrating serial dilutions of the culture supernatants onto target cells (TZM-bl [12] and DFJ8 [2] cell lines for HIV and MLV, respectively). Luciferase activity was measured 36 to 48 h postinfection in TZM-bl cell lysates was measured by titering serial dilutions of the culture supernatants onto target cells (TZM-bl [12] and DFJ8 [2] cell lines for HIV and MLV, respectively). Luciferase activity was measured 36 to 48 h postinfection in TZM-bl cell lysates. The experiment was carried out with three replicates per trial on two separate days. The data were normalized to virus infectivity released from samples transfected with empty vector and presented as infection of infectious virus released. In parallel, triplicate samples of cells and culture supernatants from the experiments described above were pooled and processed for Western blot analysis and probed with antibodies to HIV capsid (NIH AIDS Reagent Program) or MLV capsid (Camden, NJ).

RESULTS

Lipid profiles of HIV and other retroviruses. In order to analyze retroviral lipids, we purified HIV and MLV from culture supernatants of chronically infected cells by ultracentrifugation through 15% sucrose cushions. This resulted in highly purified MLV particles lacking detectable microvesicles (see Fig. S1 in the supplemental material). HIV preparations isolated from culture supernatants of H9 cells (T-cell line) and MDMs still contained nonviral particles and were further purified using anti-CD45 immunodepletion to remove these microvesicles (55) (see Fig. S1 in the supplemental material). To reveal the potential enrichment or exclusion of lipids in virus budding, we analyzed the lipids present in the plasma membrane of HIV and MLV particles from uninfected MDMs mostly share the lipid composition of HIV released from these cells infected with a mutant found that the lipid composition of HIV was not changed in the absence of the viral envelope glycoprotein (Env) (see Fig. S2A, B, and C in the supplemental material), suggesting that even though the Env protein is thought to associate with lipid rafts (46), Env does not assist the specific incorporation of any lipid. Instead, our data suggest that Gag alone dictates the major lipid components incorporated into HIV, consistent with the fact that Gag alone is responsible for lipid raft association (3) and is sufficient for particle assembly and release (33).

HIV derived from H9 cells or MDMs were also virtually identical, with the exception that dhSM is elevated in HIV from MDM cells (Fig. 1A). While the differences are subtle, they could be a consequence of the precise site of assembly in both cell types. HIV from H9 cells buds at the plasma membrane, while HIV originates at deeply invaginated membrane structures that appear to be derived from the plasma membrane in MDMs (11, 27, 57). Their overall similarity in virion lipid composition is in agreement with the emerging evidence that both assembly sites are continuous with the plasma membrane (11, 27, 47, 57). Microvesicles released from uninfected MDMs mostly share the lipid composition of HIV released from these cells but interestingly had very low levels of phosphoinositides, while HIV from the same cells contained these species (Fig. 1A). Lipid profiles of MLV from REFs showed remarkable similarity to that of HIV even with the differences in virus species and cell type (Fig. 1A and B and 2B).

The lipid composition of retroviruses resembles that of plasma membranes. The lipid composition of the plasma membrane is distinct from that of other cellular membranes, exhibiting higher levels of cholesterol and sphingolipids (56). Because HIV and MLV both use the plasma membrane for budding, we analyzed the lipids present in the plasma membrane rather than total cellular lipids to better assess enrichment. We enriched the plasma membrane via the use of cationic silica beads that adhere electrostatically to the plasma membrane (29, 53). To monitor the efficacy of these cationic beads for plasma membrane preparation, we followed enrichment of raft (flotillin and caveolin) and nonraft markers (transferrin receptor [TrF]) (Fig. 2A). Actin (cytoplasmic protein) and Rab5 (endosomal protein) served as indicators for plasma membrane purity (Fig. 2A). Membranes are relatively plastic and can be altered by outside agents. Therefore, before proceeding, we investigated whether the concentration of cationic beads had any artificial effects on the membrane preparations that could lead to artifacts. At a low bead concentration of 1%, all three markers were present to similar extents in adherent
REFs (Fig. 2A). TrF levels in the plasma membrane fractions appear to be constant with increasing bead concentration and less abundant than the total membrane levels. The fact that TrF recycles between plasma membrane and endosomes in a cell (22) explains why REF plasma membrane preparations will contain less transferrin than observed in the REF total membrane preparations. Surprisingly, the raft markers flotillin and caveolin appeared to be enriched over the nonraft marker TrF with increasing amounts of beads used, suggesting that these beads have a propensity for inducing lipid raft fractions at high concentrations (Fig. 2A). Therefore, we decided to apply the 1% cationic bead solution, which does not induce this artifact, to prepare plasma membranes from all the other adherent primary cell lines, such as MDMs. We also found that the condition works well for the suspension cell line H9, which showed equal levels of transferrin and flotillin (Fig. 2A).

Unbiased lipid profiling of purified viral particles and plasma membrane fractions revealed that the MS patterns of

<table>
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FIG. 1. Lipid composition of retroviruses in comparison to the lipid composition of total membranes of producer cells. (A) Lipid composition of different retrovirus envelopes and microvesicles (MV) produced from various cell types as described in the text. Values are expressed as molar percentages of a given lipid to the total lipid measured (except cholesterol and GM3, which were normalized to the total PC and SM signal intensities, respectively). PA, phosphatidylethanolamine; n/a, not available. (B) Ratio of retroviral lipids to total membrane lipids of host cells. Lipids that are significantly enriched (>1.5-fold) or reduced (<1.5-fold) in viral envelopes are highlighted in red and green, respectively. Each experiment was performed at least three times (n ≥ 3). Values that were significantly different (P < 0.05) from total membranes of producer cells are indicated by an asterisk.
FIG. 2. The lipid composition of retroviruses resembles that of plasma membranes. (A) Plasma membrane (PM) fractions were isolated using cationic beads. Western blots with sera against raft (flotillin and caveolin) and nonraft markers (transferrin receptor [TrR]) were used to assess the quality of the plasma membranes, while actin (cytoplasmic protein) and Rab5 (endosomal protein) served as indicators for the purity of the plasma membranes. Conditions equivalent to 1% bead solution were used for the determination of lipid contents. TM, total membranes; −, no beads. (B to G) Time of flight ESI-MS (negative mode) are shown for lipid mixtures from MLV (B), REF plasma membrane (D), and REF
MLV and HIV-H9 lipids were highly similar to their plasma membrane lipids, but not total cellular lipids (Fig. 2B to G). Both virus spectra were dominated by PS ions, namely, PS 36:1, PS 34:1, and PS 38:3 or PS 38:2 in the negative polarity ESI mode (Fig. 2B and C). This can be attributed to the abundance of PS in the lipid extract and the high ionization efficiency of PS as well as the exclusion of PI in viral envelopes. In addition to PS, other minor ions in the mass spectra corresponded to numerous species of PE, pl-PE, and GM3 which are not labeled in the figures. REF and H9 plasma membrane spectra were also similar to the viruses in having high PS levels but differed in having higher levels of PI (Fig. 2D and E). The results of the detailed analysis of fatty acid side chains is presented in Fig. 3 for HIV-H9 and in Fig. S3 and S4 in the supplemental material for MLV and HIV-MDM, respectively, indicating that retroviral envelopes have a tendency to harbor the shorter and more saturated fatty acyl chains of the cell. This distribution of retroviral lipids is highly similar to the plasma membrane while being distinctly different from the total membrane of their respective host cell (Fig. 4A and Fig. 3) (see Fig. S3 and S4 in the supplemental material).

The resulting comparison of viral lipids to plasma membrane lipids illustrates the striking similarity of retroviruses with plasma membrane lipids (Fig. 1B). In contrast, when total cell lipids are used for comparison, PS, pl-PE, SM, and dhSM, are significantly enriched, while PI, PC, PE, and Cer are reduced in viral envelopes (Fig. 4B). Thus, the plasma membrane is the appropriate cellular reference for comparison. Cholesterol, Cer, and GM3 remain enriched in both retroviral envelopes and microvesicles over their host plasma membrane (Fig. 4B) (see Fig. S4 in the supplemental material). More significantly, PIP and PIP$_2$ are enriched in the retrovirus envelopes compared to the plasma membranes (Fig. 2H) and microvesicles (see Fig. S4 in the supplemental material). These data demonstrate that while retroviral and microvesicle lipids resemble plasma membrane lipids, the presence of phosphoinositides distinguishes retroviruses from microvesicles.

The incorporation of PIP$_2$ into HIV is reduced in HIV lacking the MA domain. We next investigated the source of PIP$_2$ enrichment in retroviral envelopes. The MA domain of retrovirus Gag protein contains a polybasic patch which is argued to interact electrostatically with negatively charged PI(4,5)P$_2$ at the membrane surface during virus assembly (9, 35, 38). To test the role of the polybasic patch in PIP$_2$ incorporation, we produced and purified VLP from HEK293 cells expressing either wild-type HIV Gag or a mutant HIV Gag lacking the polybasic globular head of MA domain but still containing the N-terminal myristylation signal (AMA HIV-Gag) and compared their phosphoinositide profiles (Table 1). Strikingly, PIP$_2$ was reduced twofold in AMA HIV-Gag VLP, near the level of microvesicles, suggesting that the MA domain of HIV-Gag is responsible for the enrichment of PIP$_2$ in the retroviral envelope. Consistent with a role of PIP$_2$ in retrovirus release, HIV and MLV release were sensitive to the lowering of PIP$_2$ levels at the plasma membrane. The enzymatically active 5-phosphatase interfered with both HIV and MLV release from HEK293 cells, with a stronger effect seen in MLV (Fig. 5A and B). While the inactive form did not affect virus release significantly, we observed that at high transfection levels, the inactive form of the enzyme also interfered with MLV release, suggesting pleiotropic side effects under these conditions (Fig. 5B). Together, these results suggest that the dependence of HIV budding on PIP$_2$ is likely due to the regulatory role of MA in HIV release.

**DISCUSSION**

With this work, we present an extensive analysis of the lipidome of HIV produced from both T cells and macrophages along with the corresponding lipid contents of the plasma membrane from which the virus buds. This study greatly expands the coverage of lipid species previously presented by Aloia et al. (1) and Brugger et al. (8), including the bioactive lipids PIP, PIP$_2$, and GM3. Additionally, we analyzed the oncoretrovirus MLV to provide a more global assessment of retroviral envelopes. We report that HIV and MLV have similar lipid composition despite being produced from different cell types. Importantly, when retroviral lipids were compared to plasma membrane lipids rather than to total cellular lipids, the lipidome of retroviruses largely resembled the composition of the plasma membrane. Thus, our data do not support the idea that HIV buds from a raft with unusual lipid composition as proposed by Brugger et al. (8). The differences in our data are clearly due to the source used for lipid comparison. Brugger et al. (8) used total lipids, which includes lipids from organelles and other membrane structures that are unlikely to support retroviral budding. In contrast, our comparison between viral envelopes and plasma membranes is more virologically relevant, given the current data that support HIV budding from the plasma membrane (11, 17, 27, 57). It is important to note that we fully recapitulate the results of Brugger et al. (8) when we analyze total cell membranes.

Because the condition of the cellular reference used for comparison with the viral envelope is crucially important, we carefully considered and tested the preparation procedure for our plasma membrane preparations. One major concern is the contamination of the plasma membrane preparations with lipids from cellular organelles which have different lipid compositions (56). Hence, the use of plasma membranes for comparative lipid analysis purpose depends critically on the purity of the plasma membrane preparations. While the method we used, cationic silica bead capture of the plasma membrane, can artificially induce lipid rafts, we carefully crafted and monitored our conditions to eliminate this possibility.

Despite the similarity between the plasma membrane and the viral envelope, retroviral lipids were still distinct from total membranes (TM) (F). The lipids extracted from CD45-depleted HIV (C), H9 plasma membrane (E), and H9 total membranes (G) were analyzed similarly. The representative spectra shown are normalized to the highest peak within the m/z range. Prominent ions which were characterized by tandem MS are labeled. (H) Enrichment of phosphoinositides in retroviral envelopes. Precursor ion scanning for m/z 241 (dehydrated inositol fragment) was used to detect PI and phosphoinositides in MLV and plasma membrane.
FIG. 3. Quantification of individual species of glycerophospholipids and sphingolipids of HIV and H9 host cells. Abundance is represented as the molar percentages (y axis) of a given lipid (x axis) to total lipid measured, except for GM3, which was normalized to the total SM levels. Lipids were extracted from purified virus (black bars), total cell membrane, and plasma membrane fractions and quantified via MS using multiple reaction monitoring. The percentages were calculated with relevant internal standards. GM3 quantification is represented in relative levels due to the lack of suitable internal standards. Sphinogolipids are presented as sphingoid base residue/fatty acyl residue.
plasma membrane lipids in a number of ways. First, retrovirus envelopes are highly enriched in raft lipids, such as cholesterol and GM3 and in most cases Cer (with the exception of HIV produced from H9 cells). The enrichment of cholesterol and GM3 comes without surprise, as it has been well established that HIV buds selectively from cholesterol-enriched (40) and glycolipid-enriched (36) membrane rafts that potentially originate during initial Gag-membrane interactions (6). Moreover, HIV has been shown to specifically control the enrichment of both these lipids through its accessory Nef protein (34, 62). However, the enrichment of Cer rather than SM or dhSM (as previously reported by Brugger et al. [8]) signifies a fundamental difference in the type of lipid raft that forms during retroviral assembly. Cer molecules dramatically change the biophysical properties of rafts. In vivo, the accumulation of Cer at the plasma membrane occurs as a result of activation and surface translocation of acid sphingomyelinase (21). Not only is Cer a strong promoter of lipid raft formation, Cer-rich rafts appear to spontaneously coalesce to form larger macrodomains or platforms through fusion (5). Cer further exerts its effects by selectively displacing cholesterol in the rafts and interfering

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<th>Table 1. Phosphoinositide composition of wild-type HIV Gag VLP, ΔMA HIV Gag VLP, and microvesicles</th>
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a Values are expressed as molar percentages, and data presented are the averages ± standard deviations for three independent experiments. b WT, wild type. c MV, microvesicles.

FIG. 4. Lipid composition of retroviruses in comparison to the lipid composition of the plasma membranes of producer cells. (A) Values are expressed as molar percentages (except for cholesterol and GM3). (B) Ratio of retroviral and microvesicle (MV) lipid composition to plasma membrane lipid composition. Lipids that are significantly enriched (>1.5-fold) or reduced (<1.5-fold) in viral envelopes are highlighted in red and green, respectively. Each experiment was performed at least three times (n ≥ 3). Values that were significantly different (P < 0.05) from plasma membrane fractions of producer cells are indicated by an asterisk.

FIG. 5. Effects of PI(4,5)P2 depletion on HIV (A) and MLV (B) release from HEK293 cells. The effect of transient expression of 5PaseIV and 5PaseΔ1 was determined by normalizing virus infectivity released from samples transfected with empty vector and presented as inhibition of infectious virus released. The ratio data were obtained by normalizing the 5-phosphatase IV (5PaseIV) to 5PaseΔ1 values. The Western blots show the levels of HIV (A) and MLV (B) Gag expression in cells and released virus detected in culture supernatants.
with the association of cholesterol binding/interacting proteins with these platforms (31, 61). Thus, Cer-enriched membrane platforms appear to function as a tool that reorganizes receptor and signaling molecules in and at the cell membrane to facilitate and amplify signaling processes.

It was recently demonstrated that Cer-enriched exosomes bud into multivesicular bodies, triggered by localized accumulation of Cer through sphingomyelinase action (54). This is consistent with the enrichment of Cer shown in our own data for both microvesicles and retroviruses. However, it appears that the level of Cer has to be carefully regulated in order to ensure continued reinfection. Interestingly, increasing Cer levels in cells by pharmacological or enzymatic means inhibits HIV infectivity (16), supposedly by inducing CD4 clustering and preventing coreceptor engagement and HIV fusion (15). It is intriguing to consider how the supposed formation of Cer-enriched macrodomains would fit into a dynamic model for the formation of microvesicle and retrovirus particles.

An association with tetraspanins represents another parallel phenomenon seen between retroviruses, microvesicles, and/or exosomes. For exosomes, the enrichment of tetraspanins, including CD37, CD63, CD81, CD82, and CD86, has been well established (13, 59). Likewise, it was shown that HIV Gag and Env colocalize with distinct tetraspanin-enriched microdomains containing CD9, CD53, CD63, CD81, and CD82 during particle assembly at the plasma membrane (11, 26, 37). At the same time, components of the cellular budding machinery, including TSG101 and VSP28, are also recruited to tetraspanin-enriched microdomains to facilitate viral budding (33, 37). In this context, it is possible that the precise regulation of plasma membrane Cer and cholesterol levels helps control the dimensions of membrane macrodomain structures required to accommodate all these membrane-associated protein structures during the retrovirus assembly and budding process.

The second distinction drawn from our lipid comparison is that retroviruses lack PI, while phosphoinositides PIP and PIP2 are highly enriched compared to plasma membrane levels. We note that it is likely that phosphatase activity may have reduced the native levels of PIP2 in the plasma membrane during isolation and resulted in a corresponding increase in PI levels. Nevertheless, the key distinguishing feature that differs between retroviruses and microvesicles is the level of phosphoinositides, particularly PIP2. Microvesicles likely reflect the PIP2 levels in the native plasma membrane, thereby supporting an enrichment of this lipid in the virions. Strikingly, we determine that the MA domain of HIV-Gag is responsible for the enrichment of PIP2. Taken together, these data strongly suggest that efficient retrovirus assembly and release depend on the electrostatic interaction between both the polybasic MA domain and divalent negatively charged PI(4,5)P2 molecules at the plasma membrane.

Unfortunately, it is not feasible to discriminate stereoisomers of PIs by MS, but the majority of PIP2 at the plasma membrane in resting mammalian cells appears to be present as PI(4,5)P2 (48). Our findings are consistent with the notion that PI(4,5)P2 acts in targeting of the HIV-1 Gag protein via an interaction with the MA domain of Gag (38) to sites at the plasma membrane (17, 27). Recent structural analysis of HIV-1 Gag and its interaction with PI(4,5)P2 strongly suggest that this interaction results in the exposure of the saturated myristic acid into the budding membrane and the equivalent flipping out of the polyunsaturated 2’-fatty acid of PI(4,5)P2, resulting in the formation of an extended lipid conformation (49). At least in the case of HIV, this interaction is specific for PI(4,5)P2, and not other phosphoinositides (49). While all retroviral Gag types possess an electrostatic surface patch on its MA component (35), it remains to be determined which precise phosphoinositide species is involved in targeting Gag for other retroviruses and cell types, in particular PI(3,4,5)P3 (23). In addition to phosphoinositides, monovalent negative lipids such as PS and glycerophosphate may also contribute to the localization of Gag molecules through electrostatic interaction (60).

While it is not clear how PI(4,5)P2 is mechanistically enriched in the virus envelope, current theories of how PI(4,5)P2 is spatially regulated may provide some clues. It is currently believed that there are two separate pool of PI(4,5)P2 in the plasma membrane. About two-thirds is believed to be electrostatically sequestered by protein buffers with clusters of basic residues, such as myristylated alanine-rich C kinase substrate, to be released only in response to specific stimuli, such as an increase in local calcium ion concentration (19). The remainder of PI(4,5)P2 is unbound and free to diffuse in the plasma membrane milieu. Thus, it is unlikely that PI(4,5)P2 is able to form concentrated spots locally on their own through random diffusion. A more likely scenario lies in the initial electrostatic interaction of the Gag molecule with PI(4,5)P2 followed by the lateral sequestering of PI(4,5)P2 due to Gag-Gag multimerization.

Besides initiating Gag assembly, further roles for PI(4,5)P2 may well be a distinct possibility. Intriguingly, PI(4,5)P2 is intimately involved in the inward and outward bending of the plasma membrane in other biological systems. During endocytosis, BAR domain proteins bind to PI(4,5)P2-rich membranes to form inward invaginations (24, 63). Conversely, during the formation of filopodia, MIM and IRSp53, proteins which contain BAR-like domains, can lead to the formation of outward bending of PI(4,5)P2-rich membranes (30). Considering the strong enrichment of PIP2 lipids in the viral envelope, we hypothesize that binding of retroviral Gag proteins to PI(4,5)P2 may contribute to the induction of membrane curvature during virus assembly and budding.

In conclusion, our experimental approach in comparing retroviral lipids to the plasma membrane represents a more relevant picture of the enrichment of lipids in the viral envelope over a comparison to total cell membrane (8). Taking a broader view, this approach should be equally useful in the study of other medically important enveloped viruses. The list of enriched lipids presented herein should make a strong case for continued investigation into their contribution toward retroviral assembly and budding. However, beyond analyzing the obvious enrichment of these lipids, it must be noted that other lipids that occur at high levels but are less enriched, such as PS, pl-PE, SM, and even PC, are nonetheless important in the formation of the native retroviral envelope and deserve further investigation as well.

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