Activation of the Inositol (1,4,5)-Triphosphate Calcium Gate Receptor Is Required for HIV-1 Gag Release

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The structural precursor polyprotein, Gag, encoded by all retroviruses, including the human immunodeficiency virus type 1 (HIV-1), is necessary and sufficient for the assembly and release of particles that morphologically resemble immature virus particles. Previous studies have shown that the addition of Ca\textsuperscript{2+} to cells expressing Gag enhances virus particle production. However, no specific cellular factor has been implicated as mediator of Ca\textsuperscript{2+} provision. The inositol (1,4,5)-triphosphate receptor (IP3R) gates intracellular Ca\textsuperscript{2+} stores. Following activation by binding of its ligand, IP3, it releases Ca\textsuperscript{2+} from the stores. We demonstrate here that IP3R function is required for efficient release of HIV-1 virus particles. Depletion of IP3R by very high affinity, or blocking formation of the ligand by inhibiting phospholipase C-mediated hydrolysis of the precursor, phosphatidylinositol-4,5-biphosphate, inhibited Gag particle release. These disruptions, as well as interference with ligand-receptor interaction using antibody targeted to the ligand-binding site on IP3R, blocked plasma membrane accumulation of Gag. These findings identify IP3R as a new determinant in HIV-1 trafficking during Gag assembly and introduce IP3R-regulated Ca\textsuperscript{2+} signaling as a potential novel cofactor in viral particle release.

Assembly of the human immunodeficiency virus (HIV) is determined by a single gene that encodes a structural polyprotein precursor, Gag (71), and may occur at the plasma membrane or within late endosomes/multivesicular bodies (LE/MVB) (7, 48, 58; reviewed in reference 9). Irrespective of where assembly occurs, the assembled particle is released from the plasma membrane of the host cell. Release of Gag as virus-like particles (VLPs) requires the C-terminal p6 region of the protein (18, 19), which contains binding sites for Alix (60, 68) and Tsg101 (17, 37, 38, 41, 67, 68). Efficient release of virus particles requires Gag interaction with Alix and Tsg101. Alix and Tsg101 normally function to sort cargo proteins to LE/MVB for lysosomal degradation (5, 15, 29, 52). Previous studies have shown that addition of ionomycin, a calcium ionophore, and CaCl\textsubscript{2} to the culture medium of cells expressing Gag or virus enhances particle production (20, 48). This is an intriguing observation, given the well-documented positive role for Ca\textsuperscript{2+} in exocytic events (33, 56). It is unclear which cellular factors might regulate calcium availability for the virus release process.

Local and global elevations in the cytosolic Ca\textsuperscript{2+} level are achieved by ion release from intracellular stores and by influx from the extracellular milieu (reviewed in reference 3). The major intracellular Ca\textsuperscript{2+} store is the endoplasmic reticulum (ER); stores also exist in MVB and the nucleus. Ca\textsuperscript{2+} release is regulated by transmembrane channels on the Ca\textsuperscript{2+} store membrane that are formed by tetramers of inositol (1,4,5)-triphosphate receptor (IP3R) proteins (reviewed in references 39, 47, and 66). The bulk of IP3R channels mediate release of Ca\textsuperscript{2+} from the ER, the emptying of which signals Ca\textsuperscript{2+} influx (39, 51, 57, 66). The few IP3R channels on the plasma membrane have been shown to be functional as well (13). Through proteomic analysis, we identified IP3R as a cellular protein that was enriched in a previously described membrane fraction (18) which, in subsequent membrane floatation analyses, reproducibly cofractionated with Gag and was enriched in the membrane fraction only when Gag was expressed. That IP3R is a major regulator of cytosolic calcium concentration (Ca\textsuperscript{2+}) is well documented (39, 47, 66). An IP3R-mediated rise in cytosolic Ca\textsuperscript{2+} requires activation of the receptor by a ligand, inositol (1,4,5)-triphosphate (IP3), which is produced when phospholipase C (PLC) hydrolyzes phosphatidylinositol-4,5-biphosphate [PI(4,5)\textsubscript{P}\textsubscript{2}] at the plasma membrane (16, 25, 54). Paradoxically, PI(4,5)\textsubscript{P}\textsubscript{2} binds to the matrix (MA) domain in Gag (8, 55, 59), and the interaction targets Gag to PI(4,5)\textsubscript{P}\textsubscript{2}-enriched regions on the plasma membrane; these events are required for virus release (45). We hypothesized that PI(4,5)\textsubscript{P}\textsubscript{2} binding might serve to target Gag to plasma membrane sites of localized Ca\textsuperscript{2+} elevation resulting from PLC-mediated PI(4,5)\textsubscript{P}\textsubscript{2} hydrolysis and IP3R activation. This idea prompted us to investigate the role of IP3R in Gag function.

Here, we show that HIV-1 Gag requires steady-state levels of IP3R for its efficient release. Three isoforms of IP3R, types 1, 2, and 3, are encoded in three independent genes (39, 47). Types 1 and 3 are expressed in a variety of cells and have been studied most extensively (22, 39, 47, 73). Depletion of the major isoforms in HeLa or COS-1 cells by small
interfering RNA (siRNA) inhibited viral particle release. Moreover, we show that sequestration of the IP3R activating ligand or blocking ligand formation also inhibited Gag particle release. The above pursuits, as well as interfering with receptor expression or activation, led to reduced Gag accumulation at the cell periphery. The results support the conclusion that IP3R activation is required for efficient HIV-1 viral particle release.

MATERIALS AND METHODS

Plasmids and reagents. Plasmids encoding Gag-green fluorescent protein (GFP) (23), Gag-hemagglutinin (HA) (28), glutathione S-transferase (GST)-IP3 sponge (a high-affinity binding fragment of IP3-R) (63), viral core LA4-3 (30), and 37°C for the incubation times indicated in the text.

posttransfection, medium was removed from the plates and replaced with Dulbecco’s modified Eagle’s medium (DMEM) containing the concentrations of the drug indicated in the text. DMSO control solutions consisted of medium and an amount of DMSO corresponding to the highest volume of stock solution used in the treatment set. Plates were returned to 37°C. For indirect immunofluorescence, samples were incubated with primary antibodies, proteins were visualized using a chemiluminescence-based detection system (Lumi-light; Roche) or an infra-red-based imaging system (Odyssey; Li-Cor Biosciences). For analysis of VLP-associated Gag plus cell lysate-associated Gag.

RESULTS

siRNA-targeted depletion of endogenous IP3R inhibits VLP release. To determine whether Gag release requires IP3R, we employed previously described targeted siRNA sequences to deplete endogenous pools of IP3R-1 and IP3R-3 from HeLa cells; these two isoforms comprise 99% of the IP3R in these cells (22). The cells were mock transfected or transfected with DNA encoding Gag-GFP 24 h after transfection with control or targeted siRNAs and incubated for an additional 48 h. Targeted siRNA reduced the steady-state level of IP3R-1 (Fig. 1) and IP3R-3 (Fig. 2) in a dose-dependent manner compared to levels in mock-treated cells or cells transfected with non-targeting control siRNA. The specificity of the siRNAs was confirmed by demonstrating that expression of nontargeted protein was not affected, e.g., that the endogenous level of IP3R-3 was not affected by siRNA targeting IP3R-1 (Fig. 1A) or that the actin level was not affected by siRNA targeting IP3R-3 (Fig. 2A).

Both of the IP3R-targeted siRNAs reduced the number of VLPs detected in the medium relative to treatment with the nontargeting siRNA (Fig. 1B, top, compare lanes 1 and 2 to lanes 3 and 4). The reduction in the number of VLPs detected in the medium of IP3R-depleted cells reflected a defect in release as Gag accumulation in these cells was not diminished (Fig. 1B and 2B, bottom panels). Analysis of Gag release efficiency indicated that under the conditions where IP3R-1 or -3 was significantly depleted, the efficiency of VLP release was reduced to 10 to 20% of the levels of control samples. The results indicate that steady-state levels of the major isoforms expressed in HeLa cells and COS-1 cells (data not shown) are required for efficient VLP release. Simultaneous transfection with siRNAs targeted to the two isoforms did not result in the same level of depletion (22) or VLP release inhibition (data not shown) for unknown reasons.

Efficient production of infectious virus requires steady-state levels of IP3R. To test whether IP3R was required for production of infectious virus particles, HeLa cells transfected with the same siRNAs targeting type 1 or type 3 IP3R as above were transfected with a construct encoding HIV-1 NL4-3 provirus...
(30) at 24 h after siRNA transfection (Fig. 3). Cells transfected with siRNA targeting Tsg101 served as positive controls. Cellular levels of targeted proteins (Fig. 3A), Gag, and Gag-derived mature capsid (CA) protein (Fig. 3B) and of virus released into the tissue culture medium (Fig. 3C) were determined by Western analysis of samples prepared as above. As with Tsg101, depletion of IP3R impaired virus production significantly. Calculation of release efficiency (Fig. 3D) indicated that IP3R depletion reduced the amount of virus that was released to 8% of the level produced by mock-treated cells (Fig. 3D, bar M) or 25% of the level produced by cells transfected with the nontargeting control siRNA (bar C), similar to the effect of Tsg101 depletion (bar T). The amount of infectious virus that was released into the tissue culture medium was determined in single-cycle MAGI assays (30, 31). As shown in Fig. 3E, IP3R depletion reduced the amount of infectious virus to ≤10% of the level produced by either mock-treated or nontargeting control siRNA-treated cells. The results indicate that a steady-state level of IP3R is required for production of infectious HIV-1 particles.

**Sequestering the IP3R ligand inhibits VLP release.** IP3R is activated following binding of its ligand, IP3 (39, 47). We therefore determined the effect on VLP release of sequestering the cytosolic pool of IP3. To achieve this, we used a previously described GST-tagged fragment (amino acids [aa] 226 to 604; the sponge) (63) that contains the ligand binding region of the IP3R-1 protein. This fragment binds IP3 with ∼1,000-fold greater affinity than the IP3R-1 protein due to a mutation of R to Q at amino acid 441 (R441Q) (Fig. 4A). The sponge exhibits the same specificity for IP3 as endogenous IP3R proteins. It distributes homogenously in the cytoplasm, competes for IP3 binding with all IP3R isoforms, and does not affect IP3 production (63). As a control, we utilized a fragment encompassing the same residues but which is defective for IP3 binding due to a different mutation (K508A). As shown in Fig. 4B, coexpression of Gag with the sponge (lanes 2 and 3) reduced VLP release in a dose-dependent manner (compare lanes 2 and 3 to lane 1). In contrast, coexpression of Gag with the nonbinding fragment did not inhibit VLP release (Fig. 4B, compare lanes 4 and 5 to lane 1). The level of intracellular accumulation of Gag was not altered, and cell viability as reflected by actin also was not affected. Analysis indicated that the efficiency of VLP release was reduced as much as 4-fold in the presence of the sponge but was not affected by the nonbinding fragment (Fig. 4C). We conclude that efficient release of Gag requires endogenous levels of IP3, the activation ligand of IP3R.

We employed confocal microscopy to examine cells coexpressing HA-tagged Gag and the IP3-binding domain (BD) fragments. Gag was detected by indirect immunofluorescence, and cells that received DNA encoding the sponge or
the nonbinding fragment were revealed by expression of GFP under the control of the internal ribosome entry site (IRES) element in the same vectors (63) (Fig. 4D1). As shown in Fig. 4D2 to D4, HA-tagged Gag accumulated in both the cell interior and at the cell periphery whether Gag was expressed alone (Fig. 4D2) or coexpressed with the nonbinding fragment (K508A) (Fig. 4D3) or the sponge (R441Q) (Fig. 4D4). This indicated that overexpression of sponge did not detectably alter steady-state Gag distribution.

Blocking formation of IP3 by inhibiting PLC activity inhibits VLP release. PLC-catalyzed hydrolysis of PI(4,5)P2 is the cellular mechanism by which IP3 is produced (16, 25). U73122 is a widely used inhibitor of this event (Fig. 5A) and has also been used to inhibit PLC activity in HIV-infected cells (21). U73343 is an inactive form of the drug that must be used as a control to ensure that observed effects are specific to inactivation of the PLC enzyme activity (25). Cells were transfected with DNA encoding Gag-GFP; at 24 h posttransfection the tissue culture medium was replaced with treatment medium containing either DMSO alone or 0.5 or 1.0 μM U73122 or U73343 in DMSO. These concentrations are significantly lower than those previously shown to result in off-target effects (>3 μM) (reviewed in reference 25). In fact, given the short half-life of U73122 in the medium (72), this initial concentration is expected to be significantly reduced within the first few hours of the 24-h treatment. The effect of drug treatment on VLP was determined. As shown in Fig. 5B, cells exposed to U73122 (0.5 μM) produced significantly fewer VLPs (lane 2) than the cells incubated with the same concentration of U73343 (lane 3) or with the DMSO carrier (lane 1). Although the possibility of off-target effects cannot be completely eliminated, the fact that the level of Gag in cell lysates was the same in U73122-, U73343-, or DMSO carrier-treated cells makes this explanation unlikely. Moreover, the level of actin was not altered, indicating that the samples contained comparable amounts of total cell protein. Analysis of VLP release efficiency (Fig. 5C) indicated that release efficiency was reduced 10-fold in the presence of U73122 compared to the levels with U73343 or the DMSO control. Examination of the treated cells by confocal microscopy (Fig. 5D) confirmed that COS-1 cells treated with U73122 (0.5 μM) produced significantly fewer VLPs (lane 2) than the cells incubated with the same concentration of U73343 (lane 3) or with the DMSO carrier (lane 1). The effect of the active inhibitor on PI(4,5)P2 accumulation was dose dependent, as indicated by the increased detection of PI(4,5)P2 in cells treated with 1.0 μM U73122 (Fig. 5D4) compared to 0.5 μM U73122 (Fig. 5D3). At the same concentrations, the inactive U73343 analog at 0.5 μM (Fig. 5D2) and 1.0 μM (data not shown) behaved like the DMSO carrier (Fig. 5D1). Under these conditions, the subcellular distribution of Gag was significantly altered. Whereas Gag puncta were dispersed throughout the cell in 75% of 30 cells treated with the DMSO carrier (Fig. 5D1) or the inactive U73343 analog at 0.5 μM (Fig. 5D2) and 1.0 μM (data not shown) behaved like the DMSO carrier (Fig. 5D1). The results indicate that active PLC is required for efficient VLP release. We infer that PLC-mediated IP3 production is necessary to maintain normal Gag localization at the cell periphery and elsewhere.

Gag accumulates in vesicles following IP3R depletion. To determine whether depletion of IP3R also altered the subcellular location of Gag, cultures prepared as described in the legends of Fig. 1 and 2 were examined by immunoelectron

FIG. 3. Efficient production of infectious virus requires steady-state levels of IP3R. HeLa cells were mock transfected (M) or transfected with control siRNA (C), siRNA targeting Tsg101 (T), siRNA targeting IP3R-1 (I) or siRNA targeting IP3R-3 (3) and 24 h later transfected with DNA encoding HIV-1 NL4-3 provirus. After an additional 48-h incubation, cells and tissue culture medium were harvested for analysis. (A to C) Western analysis for endogenous cellular Tsg101, IP3R-1, IP3R-3, and actin (A) and for Gag and Gag-derived mature capsid (CA) proteins in the cell (B) and in the tissue culture medium (C). (D) Virus release efficiency. (E) Amount of infectious virus in the tissue culture medium determined by single-cycle MAGI assays.
microscopy. Figure 6 shows images of cells that had been transfected with nontargeting control (panels A to D) or targeted (panels E, F, and H) siRNAs. Figure 6G shows Gag, detected by Western analysis, in VLPs isolated from the medium of the control and targeted samples and confirms that the targeted siRNA inhibited VLP production. Cells treated with control siRNA were generally indistinguishable from untreated cells (Fig. 6A and data not shown). Large numbers of vesicular structures characterized by a relatively consistent diameter of ~500 nm were detected in the cytoplasm of cells treated with siRNA targeted against expression of either IP3R-1 (data not shown) or IP3R-3 (Fig. 6E; shown at higher magnification in F). Anti-CA antibodies and gold-tagged secondary antibodies were employed to identify the viral protein in postembedded thin sections. No signal was detected in “untouched” or mock-transfected cells (data not shown). In cells treated with the control siRNA, gold particles were detected throughout the cytoplasm in a manner previously described (42), i.e., in small clusters of 1 to 5 gold particles scattered throughout the cytoplasm but not associated with any apparent structure (Fig. 6B) and near the plasma membrane (panel C). None of the 500-nm vesicles in examined sections had gold particles (Fig. 6D). In contrast, in cells transfected with siRNA targeted to IP3R-1 (data not shown) or IP3R-3 (Fig. 6H), a high percentage of the gold particles was located in the vesicular structures, as shown in two independent experiments that examined 40 cells (Table 1). The results indicate that depletion of IP3R altered the subcellular location of Gag and suggest that the inhibitory effect of IP3R depletion on VLP release (Fig. 1 and 2) relates to this mislocalization.

To obtain biochemical support for the notion that Gag was localized in a different membrane compartment following depletion of IP3R, we prepared membrane-enriched fractions (P27 and P100, the pellet fractions obtained by centrifugation at 27,000 × g and 100,000 × g, respectively) as previously described (18) from siRNA-transfected HeLa cell cultures prepared as described in the legends of Fig. 1 and 2 and examined them for Gag (Fig. 7). Markers of the early endosome and lysosome, early endosome antigen 1 (EEA1) and cathepsin D,
respectively, sedimented in the P27 fraction. The plasma membrane marker sodium-potassium ATPase and mannose-6-phosphate receptor (M6PR), a marker of Golgi apparatus-derived vesicles, and MVB were detected mainly in the P100 fraction (18). As described above, lysates from cells transfected with control or targeted siRNAs typically contained comparable amounts of Gag (Fig. 7A; compare with Fig. 1B and 2B). As Gag in P27 and P100 fractions represented a very small percentage of the total Gag in the lysate, electrophoresis of the entire P27 and P100 pellet was necessary for Gag detection. Consistent with earlier observations (18), in all cases more Gag was detectable in the P27 fraction than in the P100 fraction. As Gag in P27 and P100 fractions represented a very small percentage of the total Gag in the lysate, electrophoresis of the entire P27 and P100 pellet was necessary for Gag detection. Consistent with earlier observations (18), in all cases more Gag was detectable in the P27 fraction than in the P100 fraction. However, in the P27 fractions, the samples derived from the siRNA control (Fig. 7B, lanes 1 to 6), contained less Gag than the P27 fractions isolated from the cells transfected with the siRNA targeted to IP3R-1 (lanes 7 to 12) or IP3R-3 (lanes 13 to 18). The amount of Gag detected in the P27 fraction was directly proportional to the amount of siRNA transfected into the cell. There were no detectable changes in the Gag content of corresponding soluble fractions (S100, from centrifugation at 100,000 × g) (data not shown). A longer exposure of the Western blot (Fig. 7C, top) revealed a redistribution of Gag in the two membrane-enriched fractions. Comparison of the Gag levels in the fractions derived from cells treated with 20 nM control indicated a relative distribution of 60% and 40% Gag in the P27 and P100 fractions, respectively (lanes 1 and 2). This is in agreement with previous observations (18). This shifted to a relative distribution of 90 to 95% (P27) and 5 to 10% (P100) Gag, respectively, in cells transfected with targeted siRNA (Fig. 7C, lanes 3 to 6). Since, as noted above, no obvious changes were apparent in S100 fractions, we surmise that the shift reflected changes in distribution of the membrane-associated Gag population. The results support the conclusion that Gag localization was altered following IP3R depletion.

FIG. 5. Inhibition of PLC-mediated PI(4,5)P₂ hydrolysis inhibits VLP release. (A) Schematic representation of U73122 inhibition of PLC catalysis of PI(4,5)P₂ hydrolysis into IP3 and diacyl glycerol (DAG). (B) COS cells transfected with DNA encoding Gag-GFP were treated with a 0.5 μM concentration of the PLC inhibitor U73122, the nonactive analogue U73343, or the DMSO carrier (C denotes control) for 24 h at 24 h posttransfection. Medium samples were analyzed by Western blotting for VLP production, and cell lysates were examined for Gag accumulation. (C) VLP release efficiency. (D) Gag-GFP-expressing cells subjected to treatment as described in panel B and examined by deconvolution confocal microscopy. Treated cells were simultaneously fixed and permeabilized with formaldehyde, glutaraldehyde, and saponin. PI(4,5)P₂ was detected by indirect immunofluorescence with mouse monoclonal antibody against PI(4,5)P₂ and TRITC-labeled anti-mouse secondary antibody (red). Cell nuclei were stained with Hoechst stain (blue). Bar, 10 μm.
To obtain additional support for this conclusion, cells in cultures treated with control or targeted siRNA were examined by confocal microscopy (Fig. 8). In preliminary experiments, we established that siGLO PPIB (Dharmacon), a fluorescent siRNA that localizes to processing (P) bodies in the cytoplasm (27), was delivered into ~75% of cells \((n = 305)\) under the transfection conditions used for the experiment, making it highly likely that the majority of the cells examined contained the desired siRNA molecules. Specific knockdown of IP3R expression by the targeted siRNA was confirmed by examination of VLPs in the medium collected from the control- and the targeted-siRNA-treated cultures (data not shown). In samples transfected with the siRNA control used in the experiments shown in Fig. 1 and 2 above, Gag-GFP was detected at the cell periphery and in the cell interior delineated by CD63 (red fluorescence), a commonly used marker of late endosomes and MVB which accumulates in the perinuclear region (32, 49). This is consistent with the results of previous studies (14, 34, 44, 48, 58). Representative cells are shown in Fig. 8A and C. In contrast, Gag-GFP was detected in the cell interior in most of the cells in cultures transfected with siRNA targeted to IP3R-1 (Fig. 8B and D), which is indicated by the location of CD63 (Fig. 8C and D). These results were highly reproducible in three independent trials where >40 cells were examined, with 90 to 100% of cells in control cultures containing Gag at the plasma membrane compared to <20% in cultures transfected with the specifically targeted siRNA. The results support the conclusion that steady-state levels of IP3R are necessary to maintain localization of Gag at the plasma membrane and prevent its sequestration in interior vesicles.

Interfering with ligand-receptor interaction prevents Gag accumulation at the cell periphery. Previous studies demonstrated that expression of the IP3R-binding protein, IRBIT, which binds the IP3 binding domain (BD), suppresses IP3R activity (2). Microinjection of antibody against IP3R has also been shown to interfere with release of \(Ca^{2+}\) from intracellular stores (12). To determine whether disrupting the IP3-IP3R interaction would interfere with Gag localization, we microinjected cells with a rabbit anti-IP3R antibody targeted to the highly conserved IP3 BD on the protein (Fig. 9A). The antibody was injected into the region above the nucleus of live COS-1 cells together with DNA encoding Gag-GFP, and the cells were examined by confocal microscopy 18 to 24 h later. Intracellular delivery of the solution containing both DNA and antibody into the region above the nucleus maximizes expression of the DNA while still allowing the antibody to reach the cytoplasm (43, 61). In preliminary studies, TRITC-labeled anti-rabbit secondary antibody detected the primary anti-IP3R antibody in the

![FIG. 6. siRNA-targeted depletion of IP3R sequesters Gag in interior vesicles: immunoelectron microscopy. Sections through HeLa cells in cultures treated with control siRNA (A to D) or siRNA targeted to IP3R-3 (E, F, and H) as described in the legend of Fig. 1 above were prepared for immunoelectron microscopy as described in Materials and Methods. Images in panels A and E are at low magnification, and those in panels B to D, F, and H are at high magnification. Panels D and H show vesicular structures in the cytoplasm of cells in cultures transfected with control or targeted siRNA, respectively. Arrows indicate Gag identified by probing with anti-CA monoclonal antibody and 12-nm gold particle-tagged goat anti-mouse secondary antibody. Panel G shows Gag in VLPs isolated from the medium of the control and targeted samples and detected by Western analysis with the anti-CA antibody. Bars, 500 nm (A, D to F, and H) and 100 nm (B and C).]

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### TABLE 1. Distribution of gold-tagged Gag in cells treated with siRNA

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Fraction</th>
<th>No. of gold particles</th>
<th>(% vesicle associated)</th>
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<tr>
<td></td>
<td>Expt 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Expt 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Nonvesicular</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Vesicular</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IP3R-1</td>
<td>Nonvesicular</td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Vesicular</td>
<td>4 (43)</td>
<td>32 (35)</td>
</tr>
<tr>
<td>IP3R-3</td>
<td>Nonvesicular</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Vesicular</td>
<td>12 (100)</td>
<td>102 (98)</td>
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<sup>a</sup> The primary antibody was anti-CA monoclonal antibody.<br>
<sup>b</sup> The primary antibody was anti-CA polyclonal antibody.
cytoplasm of injected cells 6 to 24 h later (data not shown). To control for off-target effects, additional samples were injected with a mixture containing DNA encoding Gag-GFP and a similar quantity of affinity-purified polyclonal antibody directed at the p85α-subunit of PI3K (Fig. 9B). As revealed by serial sectioning in the z-plane, Gag was mostly at the cell periphery (z of 0 μm) (Fig. 9B1) with very few Gag-containing structures detected in the cell interior (z of 2.4 μm) (Fig. 9B2). In contrast, when co-injected with the anti-IP3R antibody (panel C), Gag was not detectable at the cell periphery (z of 0 μm) (Fig. 9C1) but was mostly in the interior of the cells (z of 2.4 μm) (Fig. 9C2). Similar results were obtained in five independent experiments representing over 500 microinjected cells. Gag was detected at the cell periphery in >90% of cells microinjected with the mixture containing the control antibody and in <20% of cells injected with the mixture containing the anti-IP3R antibody that recognizes the IP3 binding site. The results indicate that interference with IP3-IP3R interaction inhibited localization of Gag at the cell periphery. Taken together with the results described above, where similar changes in Gag localization were observed following IP3R depletion or inhibition of IP3 formation, these studies support the conclusion that the steady-state level of IP3R and activation of the receptor are required for productive accumulation of Gag near or at the plasma membrane.

**Thapsigargin stimulates VLP release.** As our results indicated that IP3R activation, i.e., release of Ca2+ from IP3R-gated stores, promotes particle production whether directed by the gag gene alone or the entire viral genome, we determined whether thapsigargin (TG) treatment stimulated VLP release (Fig. 10). TG is a cell-permeable agent that inactivates the Ca2+ ATPase pump in the ER membrane, effectively causing a rise in cytosolic Ca2+ (26, 36). COS-1 cells were transfected with DNA encoding Gag-GFP, treated with thapsigargin or the DMSO carrier in calcium-free, serum-free DMEM 24 h later, and harvested following a 4-h incubation period. Treatment under the calcium-free medium condition eliminates Ca2+ influx effects (10, 51), allowing evaluation of the effect of release of stored Ca2+ alone. However, this condition restricts the refilling of the ER, which limits the rise in cytosolic Ca2+ above its basal level. At the end of the 4-h incubation period, the medium and cells were harvested for analysis of VLPs and cell-associated Gag. As shown in Fig. 10A, the cells treated with thapsigargin exhibited a dose-dependent increase in the amount of VLP detected in the medium (lanes 2 to 4) compared to cells incubated with the DMSO carrier (lane 1). In contrast, the accumulation levels of cell-associated Gag and actin remained constant. Analysis of the results (Fig. 10B) indicated that thapsigargin stimulated a small but reproducible enhancement in VLP release (i.e., a 4-fold increase in VLP release efficiency at the highest dose). The results indicate that...
release of the intracellular Ca\(^{2+}\) pool stored in the ER promotes VLP release. As the thapsigargin-sensitive Ca\(^{2+}\) pool is the IP3-responsive Ca\(^{2+}\) pool (65), we can interpret this result as indicating that release of the IP3R-gated Ca\(^{2+}\) pool in the ER promotes VLP release.

To examine the effect of thapsigargin on Gag localization, the cells were examined by confocal microscopy. As shown in Fig. 10C, Gag was detected at the cell periphery (z of 0 µm) (C1, top) and in the cell interior (z of 1.2 µm) (C1, bottom) in cells treated with the DMSO carrier, as revealed by serial sectioning in the z plane. In contrast, Gag accumulation in the cell interior was significantly reduced in cells treated with thapsigargin (compare Fig. 10C2 top and bottom panels). As shown in Fig. 10D, and consistent with the results shown above (Fig. 9), Gag localized with CD63 in the perinuclear region in the cells treated with DMSO (Fig. 10D1). However, this localization was significantly reduced in thapsigargin-treated cells, as evident by inspection of the interior z section (Fig. 10D2, panels).
bottom). Instead, Gag accumulated almost exclusively at the cell periphery (Fig. 10D2, top). We conclude that release of the IP3R-gated Ca\(^{2+}\) pool from the ER minimizes Gag localization in the cell interior and promotes Gag release.

### DISCUSSION

Previous studies showed that elevation of cytosolic Ca\(^{2+}\) by addition of CaCl\(_2\) and ionomycin to the tissue culture medium enhances Gag release efficiency (20, 48). Our studies demonstrate that IP3R, a cellular factor that gates intracellular Ca\(^{2+}\) stores, is required for efficient Gag trafficking and virus particle release. We showed that experimental conditions that block Ca\(^{2+}\) release through IP3R channels on the ER, i.e., depletion of IP3R or its activating ligand, inhibited VLP release. In contrast, VLP release was stimulated following thapsigargin-induced mobilization of the IP3R-gated Ca\(^{2+}\) pool from ER stores. Examination of cells by confocal or electron microscopy indicated that interference with the IP3-IP3R interaction by all of the experimental strategies we employed except the sponge inhibited Gag accumulation on the plasma membrane. In contrast, more Gag localized at the cell periphery after thapsigargin treatment. Perhaps the stoichiometric nature of sponge-IP3 interaction (i.e., 1:1), combined with the fact that IP3 is continuously generated in the cell, will always result in IP3 levels exceeding that of the sponge, and this may make sponge impact difficult to detect visually. Nevertheless, the results of our study indicate that IP3R is an important cofactor of Gag trafficking and infectious virus production.

The plasma membrane is the predominant accumulation site of the acidic phospholipid PI(4,5)P\(_2\) (70). Gag is targeted to the plasma membrane by PI(4,5)P\(_2\), and this event is required for viral particle release (45; reviewed in reference 69). The “hydrolysis stimulating synthesis” model postulates that PI(4,5)P\(_2\) turnover is required to maintain the supply of plasma membrane PI(4,5)P\(_2\). This model states that hydrolysis and
synthesis of PI(4,5)P2 are tightly coupled events, such that synthesis rapidly compensates for its hydrolysis while PI(4,5)P2 hydrolysis sends signals that stimulate its production (35). As all four isoforms of PLC require Ca2+ for catalytic function (25, 54) and as Ca2+ has been found to increase both the level and activity of PIP5K1c, the lipid kinase that is critical for synthesis of PI(4,5)P2 (74), Ca2+ is a key regulator of this dynamic process.

Our results suggest that PI(4,5)P2 turnover also is critical for maintaining Gag at the plasma membrane. We observed that treatment of cells with U73122, an inhibitor of PLC-mediated PI(4,5)P2 hydrolysis, resulted in accumulation of Gag in the cell interior and inhibition of VLP release even though the inhibitor was added 24 h posttransfection when a significant amount of Gag was already plasma membrane bound, as revealed by the control samples. This finding suggests that plasma membrane association of Gag, driven by membrane binding and targeting determinants (55, 59, 71), requires the dynamic relationship between turnover and synthesis of plasma membrane PI(4,5)P2. The fact that interfering with IP3R activation by several different approaches resulted, in almost all cases, in accumulation of Gag in the cell interior rather than at the plasma membrane suggests that Ca2+ signaling, mediated through IP3R activation, functions to oppose the Gag trafficking associated with this interior localization.

In general, cellular cargo destined for endocytic trafficking is tagged with ubiquitin to facilitate recognition by Tsg101 and other ESCRT factors (reviewed in reference 52). Tsg101, which binds HIV-1 Gag directly (17, 37, 38, 67), has been reported to increase the level of ubiquitinated Gag (41). As noted above, MVβ-associated Gag has been reported by several laboratories (14, 34, 44, 46, 48, 58). Under conditions of impaired IP3R function, we found Gag associated with MVβ-like vesicles. Studies by Mullock et al. (40) provide evidence for formation of an MVβ-immature lysosome hybrid which eventually generates a mature hydrolytic lysosome and a re-formed late endosome. The former was suggested to contain proteins that were sorted into intraluminal vesicles for degradation; the reformed late endosome contained proteins on a nondegradative pathway. In their model, calcium is the determinant of the sorting (50). If ubiquitination signaling can be considered an “agonist” of Gag endosome association, we hypothesize that Ca2+ serves as an antagonist of this event.

There are two possible sources of intracellular Ca2+ that might mediate the effects on VLP production that we observed. It is known that IP3R-mediated release of Ca2+ results in an initial, transient stimulus that is followed by a second, slower Ca2+ wave that is mediated by store-operated channels (SOCs) in the plasma membrane (reviewed in reference 51). Channel activity is coupled to IP3R activity (4, 57, 62). One of these channels, G protein-coupled receptor extracellular Ca2+ (Ca2+,o)-sensing receptor (CaR) (62) links IP3R-mediated Ca2+ signaling and ubiquitin-mediated signaling. CaR binds directly to a deubiquitinating enzyme (24) that is known to interact with ESCRT proteins and whose activity is required for retroviral budding (1). Through its ability to influence proteins like CaR, IP3R might regulate the ubiquitination state of plasma membrane-localized Gag, permitting the viral protein to counter nonproductive sorting signaled by polyubiquitination. If Gag ubiquitination is controlled by L domain binding partners as reported for Tsg101, in the case of HIV (41), or Nedd4, in the case of avian sarcoma virus (ASV) (64), the model predicts that these proteins would play a critical role in linking Gag to the machinery controlled by IP3R.

Mobilization of Ca2+ from intracellular storage pools is now a recognized factor in HIV replication (11). Nef-mediated T-cell activation, Tat-dependent HIV-1 gene expression, and gp120-mediated events are steps in virus replication for which Ca2+ mobilization is known to be important. Previous studies demonstrated that extracellular Ca2+ enhanced VLP release

![FIG. 10. Thapsigargin stimulates VLP release. (A) COS-1 cells expressing Gag were incubated for 4 h in calcium-free, serum-free DMEM containing either DMSO carrier alone (lane 1) or thapsigargin (lanes 2 to 4). Tissue culture medium and cells were harvested for analysis of VLPs and cell-associated Gag and actin. (B) Analysis of VLP release efficiency. (C and D) Examination of cells by deconvolution confocal microscopy. DMSO-treated control cells showing Gag at both the cell periphery (C1 and D1, top) and in the cell interior (C1 and D1, bottom). Thapsigargin-treated cells show Gag at the cell periphery (C2 and D2, top) but not, or at reduced levels, in the cell interior (C2 and D2, bottom). CD63 was detected by indirect immunofluorescence with mouse mononclonal anti-CD63 antibody and TRITC-tagged secondary antibody (red). Cell nuclei were stained with Hoechst stain (blue). z, section through z plane of the cell. Bar, 10 µm.]
Our study links, for the first time, HIV-1 Gag trafficking and assembly with mobilization of Ca$^{2+}$ from intracellular stores gated by a specific Ca$^{2+}$ channel receptor. Gag itself or any of the proteins that Gag recruits may serve as a physiologic trigger of IP3R activation for delivery of Ca$^{2+}$ at spatially and temporally appointed events in the Gag release pathway. Whatever the trigger, our findings identify IP3R as a new determinant in HIV-1 Gag trafficking and assembly and

FIG. 10—Continued.
introduce IP3-regulated Ca\(^{2+}\) signaling as a potential novel cofactor in viral particle release.

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REFERENCES


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