Role of the Human T-Cell Leukemia Virus Type 1 PTAP Motif in Gag Targeting and Particle Release

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Human T-cell leukemia virus type 1 (HTLV-1) Gag is targeted to the plasma membrane for particle assembly and release. How HTLV-1 Gag targeting occurs is not well understood. The PPPY and PTAP motifs were previously shown to be involved in HTLV-1 particle release with PTAP playing a more subtle role in virus budding. These L domains function through the interaction with host cellular proteins normally involved in multivesicular body (MVB) morphogenesis. The plasma membrane pathway rather than the MVB pathway was found to be the primary pathway for HTLV-1 particle release in HeLa cells. Intriguingly, disruption of the PTAP motif led to a defect in the targeting of Gag from the plasma membrane to CD63-positive MVBs. Particles or particle buds were observed to be associated with MVBs by electron microscopy, implying that Gag targeting to the MVB resulted in particle budding. Blocking clathrin-dependent endocytosis was found not to influence localization of the HTLV-1 Gag PTAP mutant, indicating that Gag did not reach the MVBs through clathrin-dependent endocytosis. Our observations imply that the interaction between Gag and TSG101 is not required for Gag targeting to the MVB. Overexpression of dynamin p50 increased particle release, suggesting that there was an increase in the intracellular transport of MVBs to the cell periphery by the utilization of the dynein-dynactin motor complex. Intriguingly, virus particle release with this mutant was reduced by 20-fold compared to that of wild type in HeLa cells, which is in marked contrast to the less-than-twofold defect observed for particle production of the HTLV-1 Gag PTAP mutant from 293T cells. These results indicate that the role of the PTAP motif in L domain function is cell type dependent.

Many retroviruses initiate particle assembly and budding at the plasma membrane where Gag proteins associate with raft microdomains of the inner leaflet of the plasma membrane (15, 34). The Gag polyprotein is the main driving force for assembly and budding. The targeting of Gag proteins to the cell membrane and stable membrane association are accomplished using a bipartite membrane binding signal consisting of the fatty acid myristate (7, 32) added cotranslationally to the N terminus of Gag in concert with a patch of basic residues within the matrix (MA) domain (12, 17, 22, 40, 41). Myristate provides a hydrophobic interaction with the lipid membrane, while the basic residues strengthen the interaction by forming the electrostatic interactions with acidic phospholipids that are enriched at the cytoplasmic face of the plasma membrane (26, 41).

The release of the nascent virions from the cell is mediated by the late (L) domain (9, 13). The PTAP-interacting protein TSG101 functions by recruiting the intracellular machinery, ESCRT complexes, to the site of assembly at the plasma membrane (13, 15, 21, 36). These ESCRT complexes are normally involved in multivesicular body (MVB) biogenesis (1, 2). The formation of MVBs, a late endosomal compartment, is characterized by the invagination of limiting membranes to bud small vesicles. Topologically, both virus budding at the plasma membrane and the vesicle budding into MVBs share striking similarities: they occur away from the cytosol, and they involve lipid microdomains, inositol derivatives, and a particular cohort of proteins such as TSG101 and ubiquitin (9, 13).

Previous studies have investigated the role of the human T-cell leukemia virus type 1 (HTLV-1) PPPY and PTAP motifs in virus budding at the plasma membrane (5, 6, 16, 20, 31, 37). Both motifs have been implicated in budding of HTLV-1 from the plasma membrane (6, 37). The PPPY and PTAP motifs function through the interaction with the Nedd4 family of proteins (E3 ubiquitin ligases) and the TSG101 protein (an E2-like ubiquitin-conjugating enzyme), respectively (5, 6, 16). The PPPY motif was observed to be more important than PTAP in particle release (6, 16, 37). However, it remains unclear what different roles these two motifs play in HTLV-1 assembly and release. It has been suggested that PPPY and PTAP act successively in the HTLV-1 assembly process to ensure proper Gag trafficking through the endocytic pathway to late endosomes for particle release (5).

Despite the view that many retroviruses recruit the cellular machinery normally responsible for MVB formation to the plasma membrane for their assembly and budding, it has been long known that in macrophages human immunodeficiency virus type 1 (HIV-1) particles accumulate in an intracellular, vacuole-like compartment (25). A number of recent reports have identified the vacuole-like compartment as a CD63-positive MVB, indicating that HIV-1 particles may be released...
into the MVB (23, 27, 28). Such observations were further extended to standard tissue culture cell lines previously believed to support budding only at the plasma membrane (23, 24, 33). The intracellularly assembled HIV-1 particles are likely released into the extracellular environment through the fusion of virus-containing compartments directly with the cell surface in an exocytic fashion (23, 24, 27, 28, 33). Such exosome-assisted release of proteins is best documented for the major histocompatibility complex class II (MHC class II) (29, 39). It is currently unclear how retroviral Gag is targeted to MVBS.

Many intracellular vesicles are transported along polarized cytoskeletal elements (i.e., microtubules) by utilizing molecular motor proteins (18). Kinesin moves towards the fast-growing end (or plus end), away from the microtubule organizing center (MTOC), and cytoplasmic dynein moves toward the minus end. In the case of dynein, vesicle binding is thought to be mediated by the interaction of the dynein intermediate chains with the 150-kDa dynactin subunit Glued. Overexpression of the 50-kDa “dynamitin” subunit of the dynactin complex can dissociate the complex (18). Under these conditions dynein is released from its cargo sites, resulting in inhibition of minus-end-directed vesicle transport. The dynaein-dynactin motor complex has been reported to be involved in the intracellular transport of the MHC class II-containing compartment (MIIIC), and these MIICs can be released as exosomes (29, 38, 39).

To date, the use of the MVB pathway for budding has mainly been reported for HIV release from macrophages. In the present study, the plasma membrane pathway rather than the MVB pathway was found to be the primary pathway for HTLV-1 particle release in HeLa cells. Intriguingly, when PTAP is mutated, Gag strongly colocalizes with CD63, an MVB marker. Particles and particle buds were observed with intracellular vacuoles, suggesting that Gag targeting to the MVB resulted in particle budding. When clathrin-dependent endocytosis was blocked, localization of the HTLV-1 Gag PTAP mutant was not altered, indicating that Gag did not reach the MVBs through clathrin-dependent endocytosis. These findings imply that the interaction between Gag and TSG101 is not required for Gag targeting to the MVB. Overexpression of dynamitin p50 increased particle release, suggesting that there is an increase in the intracellular transport of MVBS to the cell periphery by the utilization of the dynein-dynactin motor complex. Virus particle release with this mutant was reduced by 20-fold compared to that of wild type (wt) in HeLa cells, which is in marked contrast to the less-than-twofold defect observed for particle production of the HTLV-1 Gag PTAP mutant from 293T cells. These results indicate that the role of the PTAP motif in L domain function is cell type dependent.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% Fetal Clone III serum (HyClone, Logan, UT). Transfection of cells was performed by the calcium phosphate precipitation method. Typically, 100-mm petri dishes were used in this study unless otherwise noted. A total of 10 μg of purified plasmid DNA was mixed with 50 μl 2.5 M CaCl₂ and 500 μl 2× HEPES-buffered saline and then added to cells that were split the day before transfection and at 50 to 70% confluence. At 12 to 15 h after transfection, medium was replaced with medium with 10 mM sodium butyrate and 20 mM HEPES. After 8 h of incubation, medium was replaced with fresh Dulbecco’s modified Eagle’s medium with 20 mM HEPES.

Plasmids. The plasmid pCMV-HT1 was derived from an infectious molecular clone of HTLV-1 by replacing the 5’ long terminal repeat (LTR) with a cytomegalovirus immediate-early promoter joined to a small fragment from the R region of the LTR that contains the major splice donor site (8). Eps-DH1-GFP (green fluorescent protein) and Eps-D3Δ2-GFP were kindly provided by Alexandre Bemerech (Développement Normal et Pathologique de Système Immunitaire, Paris, France). The dynamitin p50 expression construct was kindly provided by Jacques Neefjes (Netherlands Cancer Institute, The Netherlands).

Antibodies. The following antibodies were used: mouse monoclonal anti-HTLV-1 p19 (Zoetemix, Buffalo, NY), goat polyclonal anti-HTLV-1 p24 (Advanced Biotechnologies, Columbia, Maryland, and Advanced BioScience Laboratories, Inc., Kensington, Maryland), mouse monoclonal anti-CD63 (BD Biosciences), mouse monoclonal anti-LAMP-1 (Southern Biotechnology Associates, Inc., Birmingham, AL), mouse monoclonal anti-EEA1 (BD Biosciences), mouse monoclonal anti-GM130 (Transduction Laboratories), mouse monoclonal anti-polyubin (Sigma, Saint Louis, Missouri), and mouse monoclonal anti-dynactin p50 (BD Biosciences). Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG), Alexa Fluor 568-conjugated rabbit anti-goat IgG, and Alexa Fluor 647-conjugated goat anti-mouse IgG were purchased from Molecular Probes (Molecular Probes, Eugene, OR). Nanogold-conjugated rabbit anti-mouse IgG (NanoProbes Inc., Yaphank, NY) was used for electron microscopy immunolabeling.

TF uptake. The transferrin (TI) uptake assay was performed using Alexa Fluor 546-labeled transferrin (Molecular Probes, Eugene, OR). HeLa cells were incubated with 0.1 μM Alexa Fluor 546-labeled transferrin at 37°C for 15 min for binding and then washed and fixed for immunofluorescence analysis.

Confocal microscopy. Cells were grown on coverslips and fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), quenched in 100 mM glycine-PBS, and blocked in PBS containing 3% bovine serum albumin. The cells were then incubated with various antibodies diluted in the blocking buffers. Cells were washed and stained with fluorescent secondary antibodies, washed extensively, and mounted on slides. Images were collected with Zeiss 510 (Carl Zeiss, Germany) and Olympus FV500 (Olympus America Inc.) confocal microscopes. Quantitation of colocalization in single-plane images was determined using Adobe Photoshop CS2 software.

Transmission electron microscopy. Transmission electron microscopy was performed as previously described (37). For thin sectioning, transiently transfected HeLa cell pellets were fixed with 2.5% glutaraldehyde. After dehydration in a grade series of cold ethanol, the samples were embedded in Poly/Bed 812 resin (Pelco International, Redding, CA). Ultrathin sections (90 nm) were then stained with uranyl acetate. The stained sections were observed with a Philips CM 12 electron microscope.

Immunoelectron microscopy. Transfected HeLa cells were fixed by 4% paraformaldehyde in PBS for 20 min at room temperature. Immunoelectron microscopy was done as previously described (14). Briefly, fixed cells were permeabilized and blocked in PBS containing 3% bovine serum albumin and 0.1% saponin for 30 min. Cells were incubated with primary antibody in the blocking buffer followed by incubation with Nanogold-conjugated rabbit anti-mouse IgG. Cells were postfixed with 1.6% glutaraldehyde in PBS followed by incubation in a silver enhancement solution for 20 min. Cells were then postfixed with a 1% OsO₄ solution for 30 min before being embedded, dehydrated, and sectioned. Thin sections were examined with a Philips CM 12 electron microscope.

Analysis of virus particle release. Methods for preparing cell and virus-like particle (VLP) lysates have been detailed previously (37). Briefly, 3 days after transfection, cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitated with anti-HTLV-1 p24 polyclonal antibody (Advanced BioScience Laboratories, Inc., Kensington, Maryland). Supernatant collected from transfected cells was subjected to ultracentrifugation at 40,000 × g for 1 h to obtain the VLP pellets. HTLV immunoblot analysis was done using an anti-HTLV p19 monoclonal antibody as primary antibody and a horseradish peroxidase-conjugated anti-mouse Ig (Amersham, Arlington Heights, IL, and Roche Applied Science, Indianapolis, IN) as secondary antibody with the ECL Western analysis kit (Amersham). The efficiency of VLP production was normalized for cell-associated Gag. Real-time quantitation of band intensities was done using the Quantity One software package with the Chemi Doc XRS Documentation System (Bio-Rad, Richmond, Calif.).
RESULTS

PTAP motif plays a role in targeting Gag to the plasma membrane. We previously observed that the PTAP motif plays a less important role in 293T cells than the PPPY motif in virus release since mutation of PTAP led to a modest reduction in particle production (60% of wild-type level) and could be replaced by PPPY or YPDL without affecting efficiency of particle production (37). We recently reported that the Gag localization pattern for the Gag mutants in 293T cells was comparable to that of wt Gag (37). In HeLa cells, we found that the Gag PTAP mutants are localized more in the perinuclear region than along the cell periphery, as is the case with the Gag PPPY mutants and wt Gag (Fig. 1A and B). We observed that approximately three-fourths of the cells expressing the Gag PTAP mutants have a perinuclear staining pattern while approximately three-fourths of the wt Gag-expressing cells analyzed had a peripheral staining phenotype. These observations indicate that the HTLV-1 Gag PTAP mutants have a Gag localization pattern distinct from that of wt HTLV-1 Gag.

To test if the mutant Gag proteins might be associated with MVBs, transfected HeLa cells were labeled for the MVB marker CD63, a tetraspanin found in late endosomes and MVBs (particularly in the membranes of the internal vesicles). A high degree of colocalization (88% ± 1%) of mutant PPPY-PTRP and CD63 was observed (Fig. 1D and 1J) and to a lower degree with wt Gag (30% ± 7%) (Fig. 1C and 1J). The PPPY-PPPY mutant was found to be similar to PPPY-PTRP Gag. The mutant Gags were also observed to have strong colocalization with another MVB marker, LAMP-1 (data not shown). Less than 1% colocalization was observed between the Gag of PPPY-PTRP or PPPY-PPPY and an endoplasmic reticulum marker (data not shown); the Golgi marker GM130 (Fig. 1E and 1H); and an early endosome marker, EEA1 (Fig. 1F and 1I). These data indicate that Gag is associated with MVBs but not with the other organelles tested.

Localization of HTLV-1 particles by electron microscopy. To visualize HTLV-1 particles, we transfected the wt Gag-expressing construct pCMV-HT1 or the PPPY-PTRP derivative into HeLa cells for analysis by electron microscopy. Cells were fixed, and ultrathin sections were prepared for transmission electron microscopy 48 h posttransfection. For wt Gag, we

FIG. 1. HTLV-1 Gag localization in HeLa cells. (A) Mutants used in this study. The wt and mutant amino acid sequences are shown for the C terminus of the HTLV-1 MA domain of Gag. The wt and mutated PPPY and PTAP motifs are indicated by letters in boldface type and are boxed in the wt. (B) Distinct localization pattern of wild-type and L domain mutant Gag. Wild type; PPPY mutant construct APPY-PTAP; or PTAP mutant constructs PPPY-PTRP, PPPY-PPPY, and PPPY-YPDL were transfected into HeLa cells. Cells were fixed and stained with anti-p19 antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG as detailed in Materials and Methods. (C to I) PTAP mutant Gag proteins colocalize with an MVB marker. HeLa cells were transfected with wt (C), PPPY-PTRP (D to F), or PPPY-PPPY (G to I). Cells were stained with anti-p24 antibody (left columns) and three organelar markers (middle columns). The markers used were CD63 (an MVB marker) (C, D, and G), GM130 (a Golgi marker) (E and H), and EEA1 (an early endosome marker) (F and I). Colocalization in the merge is indicated in yellow. J. Quantitation of colocalization. Colocalization of CD63 and Gag was determined from 10 representative cell images.
observed large amounts of HTLV-1 particles budding at the plasma membrane, as we previously observed with 293T cells (37). For the PPPY-PTRP mutant, we found that the transfected cells had intracellular compartments containing what appeared to be HTLV-1 particles (Fig. 2A). Similar to the plasma membrane particles, the intracellular particles contained a spherical, electron-dense core and were of 100 to 110 nm in diameter, which corresponds to the size of authentic HTLV-1 particles (Fig. 2E). Virus particles were not readily observed in MVBs for wt Gag and for the APPY-PTAP mutant (data not shown), though this does not preclude the possibility that particles are released into the MVB.

The accumulation of HTLV-1 virus particles inside the lumen of MVBs for the PPPY-PTRP mutant could be explained by three possible mechanisms. First, the newly assembled virions could enter the lumen of MVBs by a budding process from the cytosol. Second, viral particles could be released by the cells on the plasma membrane and then reinternalized and targeted to these compartments via endocytosis, a phenomenon reported previously for HTLV-1 (30). Third, MVB-associated virus may have begun the assembly process at the plasma membrane but ended up in MVBs via membrane recycling. Further examination of ultrathin sections of preparations of transfected HeLa cells revealed immature ring-like particles inside the lumen and the budding profiles of immature particles (Fig. 2B to D). This observation supports the hypothesis that Gag protein assembled at the limiting membrane of MVBs.

Immunoelectron microscopic analysis was performed to further test the origin of these intracellular HTLV-1 particles. Labeling of particles with p19 found within MVB structures was observed (Fig. 2F). The particles appeared to also be labeled with CD63 (Fig. 2G), suggesting that these particles incorporated CD63 into their membrane as they assembled at the limiting membrane of MVBs.

Blocking endocytosis does not influence HTLV-1 Gag PTAP mutant distribution. The possibility of endocytosis was further excluded by examining the effect of overexpression of an en-
docytic mutant on the distribution of the PPPY-PTRP mutant Gag. The rationale for this is that, if the Gag ended up in the MVBs through the endocytic pathway, blocking the endocytosis will prevent the accumulation of Gag inside MVBs. Eps15 (epidermal growth factor receptor pathway substrate clone 15) is a constituent of plasma membrane clathrin-coated pits. Overexpression of a dominant-negative mutant of Eps15, Eps-DIII-GFP, but not another mutant, Eps-D3/H90042, was shown to inhibit the clathrin-dependent endocytosis by inhibiting the clathrin-coated pit assembly (3, 4). The inhibitory effect of Eps-DIII-GFP on endocytosis was first tested with transferrin. HeLa cells were transfected with the Eps-DIII-GFP or Eps-D3/H90042-GFP construct. Transfected cells were incubated with Alexa Fluor-547-labeled Tf, a small molecule that is normally taken up into cells through clathrin-mediated endocytosis. Cells were fixed and then analyzed by confocal microscopy. The distribution of Eps-DIII-GFP was mainly cytosolic (Fig. 3A) as reported previously (3). The punctate staining of Tf indicated that Tf molecules were taken up by cells through endocytosis and localized in intracellular vesicles in the cells that did not express Eps-DIII-GFP (Fig. 3A). However, the cells expressing Eps-DIII-GFP (Fig. 3A) dramatically reduced the uptake of Tf. In contrast, overexpression of Eps-D3Δ2-GFP did not inhibit the endocytosis of Tf into HeLa cells (Fig. 3A). The construct Eps-DIII-GFP was then used to test the effect of DIII overexpression on Gag localization. HeLa cells were co-transfected with either wt, PPPY-PTRP, or PPPY-PPPY and Eps-DIII-GFP. Cells were then stained for Gag and CD63 and analyzed by confocal microscopy. In the cell expressing DIII-GFP, wt Gag was observed to colocalize at low levels with CD63 as indicated by the merged light pink color (Fig. 3A). Strong colocalization was observed with the PPPY-PTRP and the PPPY-PPPY Gag mutants (Fig. 3C and D). The punctate staining of Gag was found to be less in these experiments when Gag was coexpressed with Eps-DIII-GFP construct. The overexpression of the Eps-D3Δ2-GFP construct with wt and mutant Gag led to results similar to that with overexpression with Eps-DIII-GFP with Gag (data not shown). Taken together,
these observations support the interpretation that Gag did not reach the MVBs through endocytosis and that the presence of virus particles or virus buds inside MVBs is the result of targeting and assembly of Gag at the MVBs. Although our data would rule out virus uptake by clathrin-dependent endocytosis, it is formally plausible that a clathrin-independent endocytic pathway could be involved in virus uptake.

**Influence of dynamitin p50 overexpression on Gag localization.** We observed that virus particles for the PPPY-PTRP mutant accumulated inside MVBs. To determine whether dynamitin p50 could influence the localization of Gag, dynamitin p50 was overexpressed together with wt Gag, PPPY-PTRP, or PPPY-PPPY. Cells were then stained with anti-p24 and anti-CD63 antibodies followed by staining with Alexa Fluor 568-conjugated rabbit anti-goat IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG. Localization of Eps-DIII-GFP (green), Gag (red), and CD63 (blue) is shown. Colocalization of Gag, Eps-DIII-GFP, and CD63 in the merged image is indicated by the light pink color.

In order to assess whether the redistribution of HTLV-1 Gag in cells in the various experiments described above would influence virus particle release from HeLa cells, we investigated particle production in the different experi-

**FIG. 3. Overexpression of an endocytic mutant does not affect association of PTAP mutant Gag with CD63. (A) Blocking of transferrin uptake.** HeLa cells were transfected with either Eps-DIII-GFP or D3Δ2-GFP followed by incubation with transferrin. The uptake of transferrin was examined by confocal microscopy. The expression of Eps-DIII-GFP or D3Δ2-GFP is indicated by green fluorescence, and transferrin location is indicated by red fluorescence. (B to D) Overexpression of a dominant-negative mutant of Eps15 does not influence Gag localization to MVBs. HeLa cells were cotransfected with Eps-DIII-GFP and either wt Gag, PPPY-PTRP, or PPPY-PPPY. Cells were then stained with anti-p24 and anti-CD63 antibodies followed by staining with Alexa Fluor 568-conjugated rabbit anti-goat IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG. Localization of Eps-DIII-GFP (green), Gag (red), and CD63 (blue) is shown. Colocalization of Gag, Eps-DIII-GFP, and CD63 in the merged image is indicated by the light pink color.
ments using an immunoprecipitation-immunoblot procedure with real-time signal acquisition. First, to help define the linear range of detection for the assay, a dilution series was made and tested (Fig. 5A). We found that the linear range of detection was at least 256-fold, based on the dilution series tested.

We next analyzed, in parallel, particle production of HTLV-1 particles with wt Gag or the PPPY-PTRP mutant. Interestingly, we observed that particle release of the PPPY-PTRP mutant from HeLa cells was about 20-fold lower than that observed with the wt Gag (Fig. 5B and C, lanes 1 and 2). This is in contrast with what we have observed with the same mutant in 293T cells, where particle production was reduced by less than twofold compared to wt. Also, the observation that the PTAP motif can play an important role in particle release is different from observations made by others (6, 20, 37). Our observations indicate that the PTAP motif plays an important role in HTLV-1 particle release and that this effect is cell type dependent. This conclusion is consistent with observations made with the HIV-1 PTAP motif (10).

Cotransfection with the dynamitin p50 was found to not significantly influence virus release for the wt but did increase (~10-fold) virus release of the PPPY-PTRP mutant (Fig. 5B and C, lanes 1 to 4). The expression of dynamitin p50 was not altered by coexpression of the different viral constructs (Fig. 5B). Based on the ability of dynamitin p50 to move MVBs away from the expected position of the MTOC, this confirms the prediction that overexpression of this construct could increase virus particle release, particularly with the PPPY-PTRP mutant.

**DISCUSSION**

The Gag protein for most retroviruses is thought to be targeted to the plasma membrane, where particle assembly and release occur. In this study, we have observed that HTLV-1 Gag can colocalize with an MVB marker. Particles or particle buds were also observed to be associated with MVBs by electron microscopy, implying that Gag targeting to the MVB resulted in particle budding. Three possible mechanisms could explain these observations. First, we have observed immature ring-like particles inside the lumen of MVB and the electron-dense Gag aggregates that are in the process of assembly or budding along with mature particles. These observations suggest that Gag was targeted to MVBs and assembled into virions inside the lumen of MVB and the electron-dense Gag aggregates that are in the process of assembly or budding along with mature particles. These observations suggest that Gag was targeted to MVBs and assembled into virions. Second, the intracellular Gag showed no obvious colocalization with an early endosomal marker, EEA1, suggesting that Gag proteins are not transported to the MVB from early or recycling endosomes, which would be the case if the virus particles were internalized through endocytosis. Third, immunogold labeling of pre-embedded cells showed HTLV-1 particles inside MVBs deco-

![FIG. 4. Influence of dynamitin p50 overexpression on Gag localization. (A) The potential intracellular transport of HTLV-1 MVB particles using the dynein-dynactin motor complex. The interaction of the MVB with microtubules leads to movement towards either the MTOC or the cell periphery by the action of the microtubule-dependent motor proteins (dynein-dynactin motor complexes) dynein and kinesin, respectively. Overexpression of dynamitin p50 is predicted to cause movement of complexes towards the cell periphery. (B to D) Overexpression of dynamitin p50 influences Gag localization. HeLa cells were cotransfected with dynamitin p50 and either wt Gag (B), PPPY-PTRP (C), or PPPY-PPPY (D). Cells were stained with anti-p50 and anti-p24 antibodies, and cells were visualized by confocal microscopy. Transfected cells were stained with anti-p24 and anti-CD63 antibodies followed by staining with Alexa Fluor 568-conjugated rabbit anti-goat IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG. (E) Quantitation of colocalization. Colocalization of dynamitin p50 and Gag was determined from nine representative cell images.](http://jvi.asm.org/
FIG. 5. Cell type dependence of the PTAP motif in HTLV-1 particle release. (A) Linear range of detection for immunoblot analysis. Protein was diluted (1:1, 1:2, 1:4, 1:5, 1:8, 1:10, 1:16, 1:32, 1:64, 1:128, and 1:256) and was subjected to immunoblot analysis. The band intensity (arbitrary units) for each dilution was determined with the Quantity One software package of the Chemi Doc XRS Documentation System (Bio-Rad) and plotted against the amount of protein used for immunoblot analysis (100, 50, 25, 12.5, 6.3, 3.1, 1.5, 0.7, and 0.3 arbitrary units) to determine if protein detection within the dilution range was linear. (B) Analysis of virus particle release. HeLa cells were transfected with wt or the PPPY-PTRP mutant either alone or with a dynamitin expression construct (as indicated). Forty-eight hours posttransfection equal volumes of supernatant medium from each culture were collected and VLPs were concentrated by ultracentrifugation. VLP production was analyzed by immunoblot analysis using an anti-HTLV-1 p19 antibody. Cell-associated material was immunoprecipitated with anti-HTLV-1 p19 prior to immunoblot analysis (see Materials and Methods). The positions of Gag and p19 are indicated. Overexpression of dynamitin p50 in cells detected by anti-p50 antibodies is shown. Quantitation of band intensities was determined by real-time acquisition of signals. Representative data from at least three independent experiments are shown. (C) Summarized data from the experiments shown in panel B.
rated with CD63, a protein that is normally found in MVB membranes. These findings suggest that HTLV-1 particles budded into the MVB, incorporating CD63 into their membrane envelope as they assembled at the limiting membrane. It should be noted that CD63 can also be found at the plasma membrane, and virion incorporation of CD63 does not exclude the possibility that CD63-containing particles were released from plasma membrane.

We found that the Gag mutant PPPY-PTRP was primarily directed to MVBs. This observation supports the hypothesis that the PTAP motif is involved in targeting Gag to the plasma membrane. This hypothesis is further supported by the observation of MVB localization of the PPPY-PPPY (Fig. 1F) or PPPY-YPDL (data not shown) mutant. In contrast, the PPPY motif does not appear to influence Gag targeting in HeLa cells since Gag of mutant APPY-PTAP was targeted primarily to plasma membrane. The role of the PTAP motif in HTLV-1 Gag targeting involves interaction with TSG101, since it was reported that overexpression of the N-terminal fragment of TSG101 redirected the particle assembly inside cytoplasmic vacuoles (6). Consistent with our findings, the role of the PTAP-TSG101 interaction in recruiting viral protein to the plasma membrane for assembly has been suggested for Ebolavirus (35). Interestingly, both HTLV-1 and Ebola virus contain both the PPPY and PTAP motifs. Another interesting observation relevant to our observations is that the deletion of the HIV-1 p6 protein of Gag (which contains the PTAP motif) does not affect HIV-1 Gag targeting to the MVB in HeLa cells and in macrophages (24). This implies that MVB targeting is not driven by an interaction between p6 and the MVB sorting machinery (e.g., TSG101).

Our study suggests that HTLV-1 particles in MVBs can be released from cells. Such an exocytic pathway has been reported in a variety of hematopoietic cell types such as B lymphocytes and dendritic cells (19, 29). In these cells, exocytosis results in the secretion of the intraluminal membrane vesicles, called exosomes. The exocytic process is best documented for the MIICs. These compartments identified as late endosomes/ MVBs have been previously shown to serve as transport organelles, which guide MHC class II to the cell surface (38, 39). Microtubules are involved in the intracellular transport of MIIC. Two molecular motor proteins, kinesin and dynein, are in control of the movement of MIIC away or toward the MTOC region (38). Our observations in this study support the conclusion that virus-containing MVBs can traffic to the cell surface and that virus release likely occurs by MVB fusion with the plasma membrane. Recent studies have suggested that AP-3 is involved in targeting HIV-1 Gag to the MVB (11). It is not currently known what role AP-3 plays in HTLV-1 Gag targeting to the MVB.

We discovered in this study that the PTAP motif can strongly influence HTLV-1 particle release. Specifically, we found that an HTLV-1 Gag mutant with an intact PPPY motif but a mutated PTAP motif (i.e., PPPY-PTRP) led to a dramatic 20-fold reduction in virus particle release. This is in striking contrast to the same mutant in 293T cells, which we previously reported to reduce particle release by less than twofold as well as not influence the distribution of Gag in cells (37). Our findings indicate that (i) PTAP function is cell type dependent and (ii) PTAP plays a role (direct or indirect) in Gag targeting to the plasma membrane. We are currently investigating why particle reduction for this mutant in HeLa cells is greatly reduced. Ono and Freed (24) observed that MVB localization of HIV-1 Gag was still observed in the absence of p6 (PTAP is located in p6), suggesting that an interaction between Gag and TSG101 is not required for Gag targeting to the MVB. This observation is consistent with our findings with HTLV-1 Gag. The observations with HIV-1 were used to hypothesize that Gag targeting involves two different cellular binding partners, one on the plasma membrane and the other in the MVB. Our current studies are directed at testing this hypothesis as well as elucidating the mechanistic basis for the cell type dependence of PTAP function.

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