Nedd4.1-mediated ubiquitination and subsequent recruitment of Tsg101 ensure HTLV-1 Gag trafficking towards the multivesicular body pathway prior to virus budding

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Summary

One of the most exciting recent developments in the field of retroviruses is the finding that their Gag proteins hijack cellular proteins from the mutivesicular body (MVB) pathway during the budding process. The Gag proteins of oncoretroviruses possess a PPxY motif that recruits a ubiquitin ligase from the Nedd4 family, whereas those of the human immunodeficiency virus interact through a PTAP motif with Tsg101, a protein of the ESCRT-1 complex. It is currently assumed that Nedd4 and Tsg101 represent equivalent entry gates towards the same cellular process leading to budding, and that both partners are recruited to the plasma membrane where viral budding occurs. However, we report here that the budding of the human oncoretrovirus HTLV-1, the Gag proteins of which possess tandem PPPY/PTAP motifs, requires both Nedd4 and Tsg101. We show that Nedd4.1, but not Nedd4.2, is recruited by the PPPY motif of Gag and subsequently catalyzes Gag ubiquitination. We also demonstrate that Gag interacts first with Nedd4.1 at the plasma membrane and then with Tsg101 in late endosomes/MVBs. Consistently, we found that HTLV-1 particles mutated in the PPPY motif remain underneath the plasma membrane, blocked at an early step of the budding process, whereas PTAP-mutated viruses accumulate in intracellular vesicles, blocked at a later step. Our findings indicate that Nedd4.1 and Tsg101 act successively in the assembly process of HTLV-1 to ensure proper Gag trafficking through the endocytic pathway up to late endosomes where the late steps of retroviral release occur.

Key words: Retrovirus, Budding, Ubiquitin-ligase, ESCRT, Multivesicular body

Introduction

The Gag polyprotein of retroviruses is the only viral protein that is both necessary for and sufficient to drive the release of virus particles through a budding process. In newly formed viral particles, Gag is cleaved by the viral protease to produce three structural core proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). The late assembly (L) domain of Gag carries the determinants required for budding. The location of this domain and its amino acid sequence vary between retroviruses (reviewed by Freed, 2002). For example, the L domains of oncoretroviruses such as Moloney murine leukemia virus (MoMuLV), Rous sarcoma virus (RSV) and Mason-Pfizer monkey virus (M-PMV) are located in a spacer peptide between the MA and CA domains and contain a conserved PPPY motif (Xiang et al., 1996; Yasuda and Hunter, 1998; Yuan et al., 1999). In lentiviruses, the L domains are contained within small proteins located at the C-terminus of Gag, p6 for human immunodeficiency viruses (HIV) (Gottlinger et al., 1991) and p9 for equine infectious anemia virus (EIAV) (Puffer et al., 1997). HIV p6 possesses a critical PTAP motif (Huang et al., 1995), whereas EIAV p9 harbors a YPDL motif (Puffer et al., 1997). Deletion of the L domains or mutations in their critical motif prevent the release of viral particles, which remain tethered to the plasma membrane of producing cells (reviewed by Freed, 2002; Pornillos et al., 2002b).

It was recently shown that retrovirus L-domains recruit cellular proteins to assist in the budding process. Hence, the YPDL sequence of EIAV p9 interacts with a subunit of the clathrin adaptor complex type 2 (AP2) (Puffer et al., 1998), which is involved in the endocytosis of plasma membrane proteins into clathrin-coated pits. The PPPY motif in RSV and M-PMV Gag mediates an interaction with a Nedd4-like protein (Kikonyogo et al., 2001; Yasuda et al., 2002), via the proline-recognition WW domains (reviewed by Macias et al., 2002). Nedd4 proteins are E3 ubiquitin ligases that, through the addition of mono-ubiquitin or short ubiquitin chains, induce endocytosis and subsequent sorting to late endosomes/multivesicular bodies (MVBs) of some plasma membrane

receptors such as the epithelial sodium channel (ENaC) (Rotin et al., 2000; Staub et al., 1997). Finally, the PTAP motif in HIV-1 p6 recruits Tsg101 (Garrus et al., 2001; VerPlank et al., 2001), a protein of the endosomal sorting complex required for transport (ESCRT), involved in the MVBs pathway (Bishop and Woodman, 2001; Katzmann et al., 2001). This process ensures the specific sorting of ubiquitinated cargo proteins into internal vesicles of late endosomes/MVBs, which result from invagination of the limiting membrane of the organelle into the lumen (reviewed by Katzmann et al., 2002).

All the cellular binding partners of L-domains described so far are linked to the trafficking of proteins along the endosomal pathway, either at early (AP2, Nedd4) or late stages (Tsg101). However, their exact implication in virus budding remains to be determined. As L-domains can be functionally exchanged between several retroviruses (Accola et al., 2000; Freed, 2002; Parent et al., 1995; Yuan et al., 2000), it is assumed that YPDL, PPPY and PTAP motifs mediate equivalent, therefore redundant, functions (reviewed by Freed, 2002). According to this hypothesis, AP2, Nedd4 and Tsg101 are possible entry points into a common pathway leading to virus release. It is also believed that viral Gag proteins recruit their cellular budding cofactors to the site of virus release at the plasma membrane (Martin-Serrano et al., 2001; Martin-Serrano et al., 2003; Pornillos et al., 2002b).

We wanted to elucidate the roles of Nedd4 and Tsg101 in retrovirus budding, and in particular to determine whether they play redundant or complementary functions. We addressed this issue by studying the budding process of the human T-cell leukemia virus type 1 (HTLV-1), the Gag protein of which possesses tandem PPPY/PTAP motifs. We report that both motifs contribute to the efficient release of HTLV-1 particles by allowing Gag to interact successively with Nedd4 and Tsg101. This allows Gag proteins ubiquitination and subsequent trafficking to late endosomes/MVBs where they could hijack the cellular machinery used for the budding of intralumenal vesicles.

Materials and Methods

Cells and antibodies

293T cells were maintained in DMEM containing 10% fetal calf serum and 2 mM L-glutamine (Invitrogen, France). HTLV-1 Gag products were detected by using serum samples from HTLV-1-infected patients. For confocal microscopy experiments, Gag proteins were detected using a purified and biotinylated anti-Gag monoclonal antibody (mAb) directed against the MA domain (Ebersold et al., 1993) (purified and biotinylated from ascitic fluid by Bioatlantic, France). Rabbit antisera to Nedd4.1 and Nedd4.2 were described elsewhere (Kamynina et al., 2001b). Anti-Tsg101 mAb was from Santa Cruz Biotechnology (Tebu, France) and FITC-conjugated anti-CD63 was from Immunotech (Coulter, France). All secondary antibodies were from Jackson Immunoresearch Laboratories (Interchim, France).

Plasmids and siRNAs

HTLV-1 proteins were expressed from the complete HTLV-1 XMT molecular clone (Derse et al., 1995), and mutagenesis of the PTAP motif (XMT-P124L, XMT-P127L) were performed by using the Kunkel procedure as previously described (Le Blanc et al., 2002; Le Blanc et al., 1999). The WW domains of Nedd4.1 were PCR-amplified from pCDN3-1-KIAA0093, which contains the entire Nedd4.1 cDNA, using a primer that introduces the Flag epitope sequence, and subcloned into pSG5. The dominant-negative mutants of human Nedd4 isoforms were generated by mutating either Cys867 to Ser for Nedd4.1 (Nedd4.1-C867S) or Cys801 to Ser for Nedd4.2 (Nedd4.2-C801S) by PCR-based techniques. Small interfering RNAs (siRNAs) homologous to Tsg101, as described by Garrus et al. (Garrus et al., 2001), and control siRNAs, not homologous to any described human gene, were produced by Genset Oligos (France).

Transfection

Transfections were performed using the calcium phosphate procedure. For immune-precipitation of viral proteins and electron microscopy experiments, 293T cells in six-well plates were transfected with 3 μg of wild-type or mutated HTLV-1 XMT proviruses, and 0.5 μg of Tax-encoding CMV-Env-delta *Pvu*II vector (Delamarre et al., 1997). For ELISA and control western blot analysis, and for detection of ubiquitinated Gag, transfections were performed in 24-well plates with 250 ng of XMT provirus, and either 250 ng (1:1) or 1 μg (1:4) of a vector encoding wild-type or mutated Nedd4 proteins, or Nedd4.1 WW domains when required. The total amounts of transfected DNA were made up to 1.25 μg using the empty pSG5 vector. For the Tsg101-depletion assay, 250 ng of provirus was transfected together with 2 μl of 20 μM solution of either the Tsg101 or the control siRNAs. For confocal microscopy, the same procedures were used except that cells were plated onto glass slides 24 hours before transfection.

Immunoprecipitation of HTLV-1 proteins and virus production

Twenty-four hours after transfection, cells were labeled overnight in medium containing 700 μCi.ml^{-1} ^{35}S Met/Cys mixture (NEN, Perkin Elmer, France). Virions were purified from cell supernatants by centrifugation on sucrose gradients. Cell- and virion-associated HTLV-1 proteins were immune-precipitated using serum from HTLV-1-infected patients (Delamarre et al., 1997).

Anti-MAp19 ELISA

Twelve hours after transfection, the culture medium was removed and the cells were incubated in 500 μl of fresh medium for 24 hours. Supernatants were harvested and cleared by a 15-minute centrifugation at 10,000 ***g*** and at 4°C. The amount of MAp19 antigen in 20 μl of supernatant was measured using the anti-MAp19 antigen ELISA from Zeptometrix (Gentaur, Belgium). To calculate the rate of virus production, the OD_{450} of supernatants of cells transfected with either a mutated provirus or with the wild-type provirus and a dominant negative construct, were compared to that of the supernatant of cells transfected with wild-type provirus and an empty vector (100% virus release, corresponding to approximately 330 pg of MAp19 antigen). We noticed that co-transfection of siRNA together with the provirus construct enhanced the mean transfection efficiency. Thus, for Tsg101 depletion assays, the OD_{450} of supernatants of cells transfected with wild-type provirus and Tsg101 siRNAs were compared with that of supernatants of cells transfected with wild-type provirus and control siRNAs.

Western blot analysis

In parallel with the ELISA, 400 μl of supernatants from transfected cells were diluted in 2 ml of ice-cold PBS containing the AEBSF protease inhibitor (Sigma), and filtered (0.45 μm pores). After centrifuging at 90,000 ***g*** for 30 minutes at 4°C, the pellets were lysed in 40 μl of lysis buffer [0.05 M Tris HCl pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, complete protease inhibitors (Roche, France)]. The cells were harvested in ice-cold PBS, then washed and lysed in 50 μl of lysis buffer. After a 30 minute incubation on ice, cell lysates were cleared by a 30 minute centrifugation at 14,000 rpm in a

microcentrifuge. Virus and cell lysates were then resuspended in 2× Laemmli Buffer (Sigma, France) and boiled for 5 minutes. Samples were subjected to 10% SDS PAGE, before being transferred onto PVDF membranes. Immunoblots were performed by using a 1:5000 dilution of serum from HTLV-1-infected patients.

To detect ubiquitinated Gag, the same procedure was used, except that harvested cells were directly lysed in 1X boiling Laemmli Buffer to avoid deubiquitination, and lysates were immediately boiled for another 10 min.

Confocal analysis

Thirty-six hours after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.05% saponin and 0.2% bovine serum albumin (permeabilizing buffer). For colocalization with endocytic markers, cells were incubated in FCS-free conditions for 30 min, and incubated for 60 min with either cyanin 3-coupled transferrin (1/100, kind gift from Dr Philippe Benaroche, Institut Curie, France) or FITC-conjugated dextran (2 mg/ml, Molecular Probes Inc, Interchim, Paris) diluted in serum-free medium prior to fixation. Gag proteins were stained using the purified and biotinylated anti-Gag mAb (5 μg/ml) and either rhodamin- or DTAF-conjugated streptavidin (2 μg/ml, Coulter, France), Nedd4.1 using rabbit anti-Nedd4.1 serum (1/500) and FITC-conjugated secondary antibodies (1/300), and CD63 with FITC-conjugated mAb (1/50). All antibodies were diluted in permeabilizing buffer. After washing, cells were mounted in Mowiol (Calbiochem, Merk Eurolab, France) and examined under a confocal microscope (model Leica TCS SP2). Images were acquired using the Leica Confocal Software (Leica, France), and processed using Adobe Photoshop.

Electron microscopy

Thirty-six hours after transfection, cells were washed in PBS and fixed for 1 hour at 4°C in a solution containing 1.6% glutaraldehyde, 1% OsO_4 and 0.1 M phosphate buffer. The cells were then rinsed three times in a solution containing 0.1 M cacodylate, incubated for 30 minutes in a 0.2 M cacodylate solution and stained for 1 hour in 2% uranyl acetate. The cells were then dehydrated in a graded series of ethanol solutions (from 25 to 100%) before being embedded in epoxy resin at 60°C for 48 hours. Ultrathin sections were cut on a Leica Ultratuc UCT microtome and then examined under a JEOL 1200EX electron microscope.

Results

The ubiquitin ligase activity of isoform 1 of Nedd4 is required for HTLV-1 Gag release

We previously reported that the PPPY motif in the MA domain of HTLV-1 Gag is required for efficient HTLV-1 particle release (Le Blanc et al., 2002). PPPY motifs from several retroviral Gag proteins recruit proteins related to the Nedd4 ubiquitin ligase E3 enzymes (Kikonyogo et al., 2001; Yasuda et al., 2002), but the precise identities of the enzymes involved have not been determined. Human Nedd4 proteins exist as at least two principal isoforms named Nedd4.1 and Nedd4.2. These isoforms are encoded by different genes (Kamynina et al., 2001b). Both proteins possess four WW domains that interact with PPxY sequences and one HECT (homologous to E6-AP carboxy terminal) domain involved in ubiquitin transfer. Nedd4.1 also contains an N-terminal C2 (Ca^{2+}-dependent lipid binding) domain (Kamynina et al., 2001b).

To determine whether Nedd4 proteins contribute to HTLV-1 budding, we used catalytically inactive mutants of Nedd4.1 and Nedd4.2, in which the active cysteine of the HECT domains had been replaced by a serine (Nedd4.1-C867S and Nedd4.2-C801S mutants). These mutants are unable to transfer ubiquitin to their target proteins, and have a dominant negative effect on their endogenous counterparts (Kamynina et al., 2001a). Anti-MAp19 ELISA (Fig. 1A, upper panel) showed that co-expression of Nedd4.1-C867S strongly interfered with

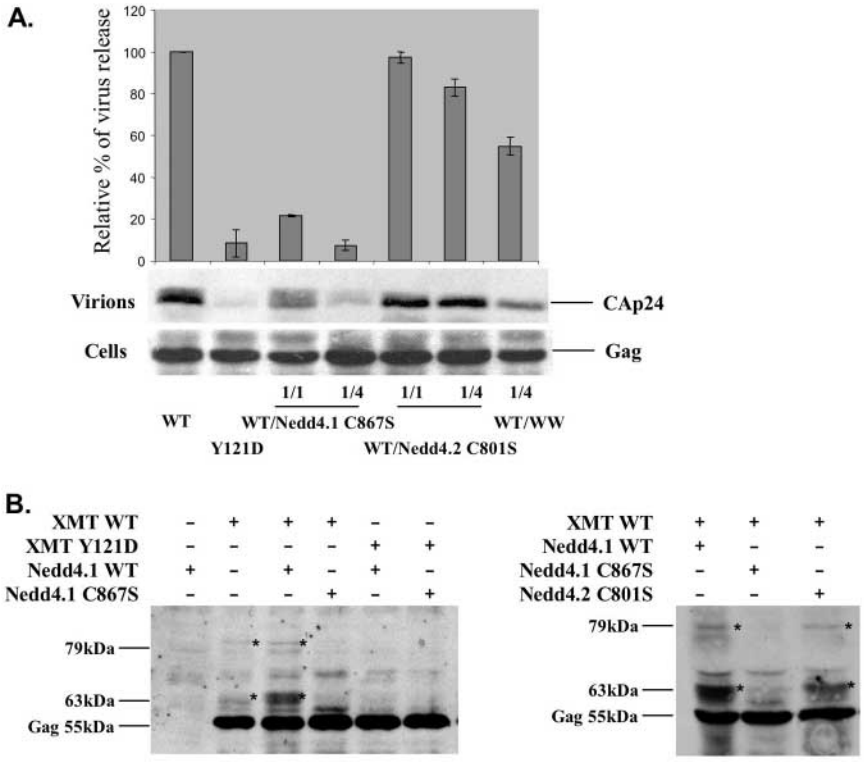

Fig. 1. Nedd4.1 is required for HTLV-1 release and catalyses Gag ubiquitination. (A) Anti-MAp19 ELISA was used to quantify virus particles released from 293T cells after co-transfection of different plasmid ratios of wild-type provirus and dominant negative mutants of either Nedd4.1 (Nedd4.1-C867S) or Nedd4.2 (Nedd4.2-C801S) proteins, or of the Flag epitope-tagged WW domains of Nedd4.1 (WW). For comparison, virus production of the PPPY-mutated provirus (Y121D) is indicated. For each condition, Gag intracellular production and extracellular virion-associated CAp24 as detected by immunoblot are also shown. The ELISA results are means and standard errors of at least three independent experiments and immunoblots correspond to one representative experiment. (B) Detection of ubiquitinated Gag products when the wild-type (XMT WT) or the PPPY-mutated (XMT Y121D) provirus is expressed alone or in combination with wild-type (Nedd4.1 WT) or dominant negative mutant (Nedd4.1-C867S) of Nedd4.1, or with the dominant negative mutant of Nedd4.2 (Nedd4.2-C801S). The position of Gag p55 is indicated, as are those of mono and tri-ubiquitinated Gag products (approximately 63 kDa and 79 kDa, respectively, asterisks).

HTLV-1 Gag release in a dose-dependent manner. Indeed, overexpression of Nedd4.1-C867S almost completely abolished virus release (90% inhibition) as did the mutation of the PPPY motif (Y121D). On the contrary, expression of Nedd4.2-C801S had only a slight effect on HTLV-1 release. Overexpression of the Flag epitope-tagged WW domains of Nedd4.1 reduced virus release by approximately 45%, consistent with the idea that the recruitment of Nedd4.1 by HTLV-1 Gag proteins involves an interaction between the PPPY motif and WW domains. Immunoblots on cell lysates (Fig. 1A, lower panel) and pelleted virions (Fig. 1A, middle panel) indicated that the level of intracellular Gag production was unaffected and that the concentrations of MAp19 antigen detected by ELISA in the supernatants of transfected cells indeed reflected the amount of virus released. These results demonstrate that the catalytic activity of a specific isoform of Nedd4 is needed for efficient HTLV-1 particle release.

Nedd4.1 catalyses Gag ubiquitination

We next investigated whether the interaction with Nedd4 proteins allows Gag ubiquitination. Cells transfected with the XMT HTLV-1 provirus were directly lysed in boiling Laemmli buffer to preclude rapid deubiquitination. This allowed the detection of at least two additional Gag products of approximately 63 kDa and 79 kDa (Fig. 1B, asterisks). Overexpression of wild-type Nedd4.1 resulted in the accumulation of these two higher molecular weight Gag products, whereas expression of Nedd4.1-C867S resulted in their disappearance (Fig. 1B). This strongly suggested that the 63 kDa and 79 kDa proteins represent mono and tri-ubiquitinated Gag, respectively. Overexpression of Nedd4.2-C801S did not affect Gag ubiquitination (Fig. 1B). Finally, ubiquitinated Gag products were not detected when the PPPY motif was mutated, even after wild-type Nedd4.1 overexpression (Fig. 1B), confirming the importance of the PPPY motif of Gag in Nedd4 recruitment.

Hence, the requirement of a specific isoform of Nedd4 for HTLV-1 budding correlates with its ability to induce Gag ubiquitination. This provides strong evidence that the role of Nedd4.1 is to trigger HTLV-1 Gag ubiquitination and that this post-translational modification is an essential step in the process of virus release.

Tsg101 and the PTAP motif of Gag are also required for efficient HTLV-1 release

A PTAP sequence is located adjacent to the PPPY motif in the MA domain of the HTLV-1 Gag proteins (Fig. 2A). To find out whether this PTAP sequence is also required for HTLV-1 release, we produced mutated XMT proviruses in which the proline-encoding codons of the motif were substituted for leucine-encoding codons (XMT-P124L, XMT-P127L, see Fig. 2A). We compared the amount of HTLV-1 released by these mutants, by the wild-type (XMT) and by the PPPY mutant (XMT-Y121D) viruses (Fig. 2B).

As previously shown, mutation of the PPPY motif strongly inhibited virus release (Fig. 2B, right panel, Y121D). We found that the P127L mutation also dramatically affected virus release, compared with the wild-type provirus (Fig. 2B, right panel, P127L), whereas intracellular Gag production was unaffected (Fig. 2B, left panel, P127L). Anti-MAp19 ELISA showed that the Y121D and P127L mutations were responsible for a 95% and 80% decrease, respectively, in virus release (Fig. 2C). Similar results were obtained for the P124L mutant (not shown). These findings indicate that as well as requiring the PPPY motif, the efficient production of HTLV-1 particles requires the integrity of the PTAP motif, revealing that this virus uses two budding motifs.

We then specifically depleted Tsg101 levels in 293T cells

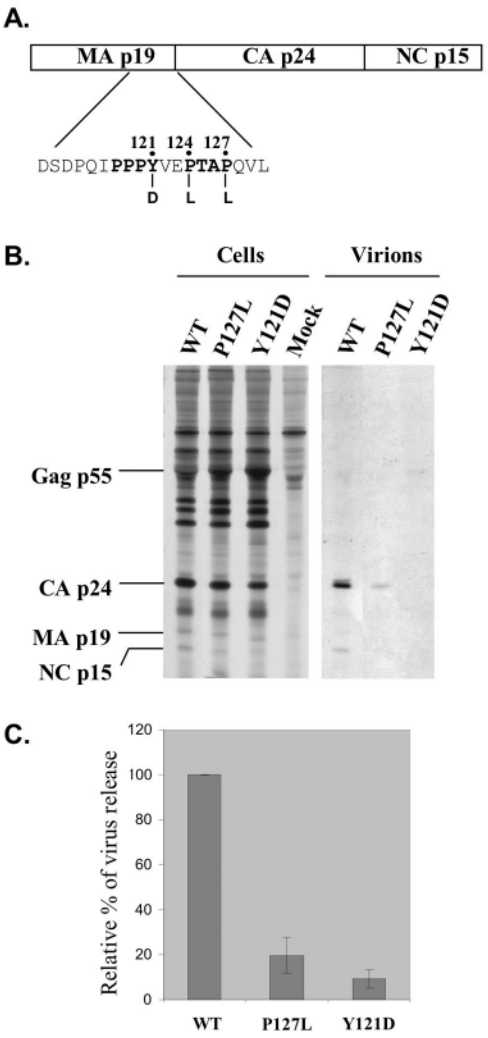

Fig. 2. The PTAP motif is also required for HTLV-1-Gag release. (A) Schematic representation of the HTLV-1 Gag protein. The sequence of the L domain at the C-terminus of the MA domain is indicated, and the PPPY and PTAP motifs are shown in bold characters. Positions of the introduced Y121D, P124L and P127L mutations are indicated. (B) Immunoprecipitation of HTLV-1 proteins from 293T cells transfected with either the wild-type (WT), PTAP-mutated (P127L) or PPPY-mutated (Y121D) provirus. Virus proteins were immunoprecipitated from either cell lysates (Cells), or from sucrose-cushion purified virions (Virions). Immature (Gagp55) and mature (CAp24, MAp19 and NCp15) Gag products are indicated. (C) Anti-MAp19 ELISA was used to quantify virus particles released from 293T cells transfected with either the wild-type (WT), PTAP-mutated (P127L) or PPPY-mutated (Y121D) provirus. The results are the means and standard errors of three independent experiments.

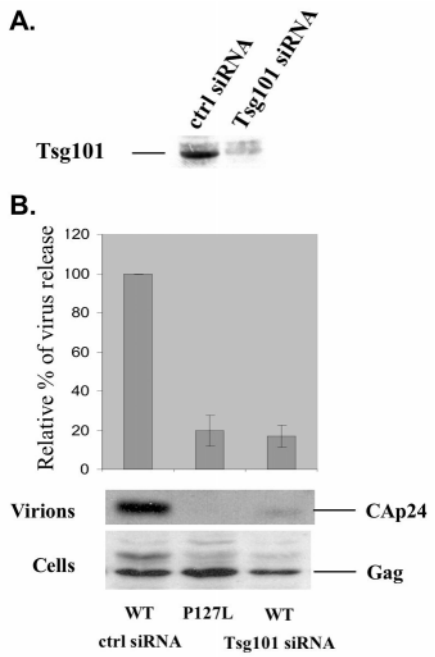

Fig. 3. Tsg101 is required for HTLV-1 release. (A) Anti-Tsg101 immunoblot analysis of 293T cells transfected with either control (ctrl) or Tsg101-specific siRNAs. (B) Anti-MAp19 ELISA was used to quantify wild-type virus release from 293T cells after depletion of Tsg101 by siRNAs. For comparison, the amount of virus particles produced by the PTAP-mutated provirus (P127L) is indicated. For each condition, Gag intracellular production and extracellular virion-associated CAp24 as detected by immunoblots are also shown. The ELISA results are the means and standard errors of at least three independent experiments, and immunoblots correspond to one representative experiment.

(Fig. 3A) and studied the effect on HTLV-1 Gag release. This treatment strongly impaired the release of HIV-1 particles (data not shown) as previously described (Garrus et al., 2001). Anti-MAp19 ELISA showed that Tsg101 depletion strongly inhibited HTLV-1 particle production and the intensity of inhibition was similar to that observed following mutation of the PTAP motif (approximately 80%, Fig. 3B, upper panel). Control immunoblots confirmed that the effect on virus release was not due to reduced intracellular Gag production (Fig. 3B, lower panel), and that levels of MAp19 antigen measured by ELISA indeed reflect the levels of virus release (Fig. 3B, medium panel). This shows that Tsg101 is involved in HTLV-1 release, strongly suggesting that HTLV-1 PTAP motif functions by recruiting Tsg101.

Wild-type Gag proteins partially localize in the endocytic pathway at steady state

We next used confocal microscopy to determine the intracellular localization of HTLV-1 Gag proteins in 293T cells transfected with the wild-type XMT provirus. Gag staining appeared as punctated structures dispersed throughout the cytoplasm (Fig. 4A-C, Gag staining). Colocalization of Gag with internalized FITC-coupled dextran indicated that a large proportion of Gag was present in the endocytic pathway (Fig. 4A). We found little, if any, colocalization of Gag with endocytosed cyanin-3-coupled transferrin, a marker of early and recycling endosomes (Fig. 4B). However, punctate Gag staining partially colocalized with the late endosomes/MVB marker CD63 (Fig. 4C). This indicates that, at a steady state in 293T cells, a significant proportion of wild-type HTLV-1 Gag proteins are present in the endocytic pathway, especially in late endosomes/MVBs.

PPPY/Nedd4.1 and PTAP/Tsg101 interactions occur at distinct intracellular sites

To characterize further the roles of Nedd4.1 and Tsg101, we determined the intracellular distribution of Gag in cells in which the interaction with one or the other of the two proteins was prevented. This was achieved by expressing either the Y121D or P127L mutated XMT provirus, or the wild-type XMT provirus in combination with either Nedd4.1-C867S or Tsg101 siRNAs.

Unlike the wild-type Gag proteins, the Y121D mutants were not dispersed throughout the cytoplasm. Instead, they appeared strongly accumulated at the plasma membrane (Fig. 5A). When the wild-type provirus was co-expressed with Nedd4.1-C867S, Gag proteins also appeared accumulated at the plasma membrane, where they colocalized with Nedd4.1 (Fig. 5B).

At first glance, mutation of the PTAP motif (P127L) did not appear to affect strongly the punctate staining of Gag (Fig. 5C, Gag staining). However, co-staining with CD63 revealed that the entire population of PTAP-mutated Gag proteins accumulated in late endosomes/MVBs (Fig. 5C, overlay). As previously reported in HeLa cells (Bishop et al., 2002), Tsg101 depletion in 293T cells modified the morphology of late endosomes that appeared to be rarer and highly vacuolated (Fig. 5D, CD63 staining). Depletion of Tsg101 from transfected cells induced the accumulation of wild-type Gag in these enlarged late endosomes (Fig. 5D).

These data indicate that functional interactions of Gag with Nedd4.1 and Tsg101 take place in distinct intracellular compartments, namely the plasma membrane for Nedd4.1 and late endosomes/MVBs for Tsg101.

Gag/Nedd4.1 interaction precedes Gag/Tsg101 interaction

The above findings either mean that Gag proteins interact with Tsg101 before they reach the plasma membrane and recruit Nedd4.1 or that Gag proteins interact first with Nedd4.1 at the plasma membrane and then come into contact with Tsg101 in late endosomes. To distinguish between these two hypotheses, we prevented Gag from interacting with both Nedd4.1 and Tsg101, and found out in which intracellular compartment Gag trafficking was arrested.

In cells that received both Tsg101 siRNAs and the Nedd4.1-C867S expression vector, wild-type Gag proteins were found at the plasma membrane (Fig. 6A), reminiscent of the localizations of the Y121D mutant virus and of the wild-type virus in cells expressing only Nedd4.1-C867S. The P127L mutant, which normally accumulated in late endosomes, was also redistributed to the plasma membrane after expression of Nedd4.1-C867S (Fig. 6B). This suggested that the defect in virus production induced by Nedd4.1-C867S occurs before the defect induced by Tsg101 depletion. Confirming this model,

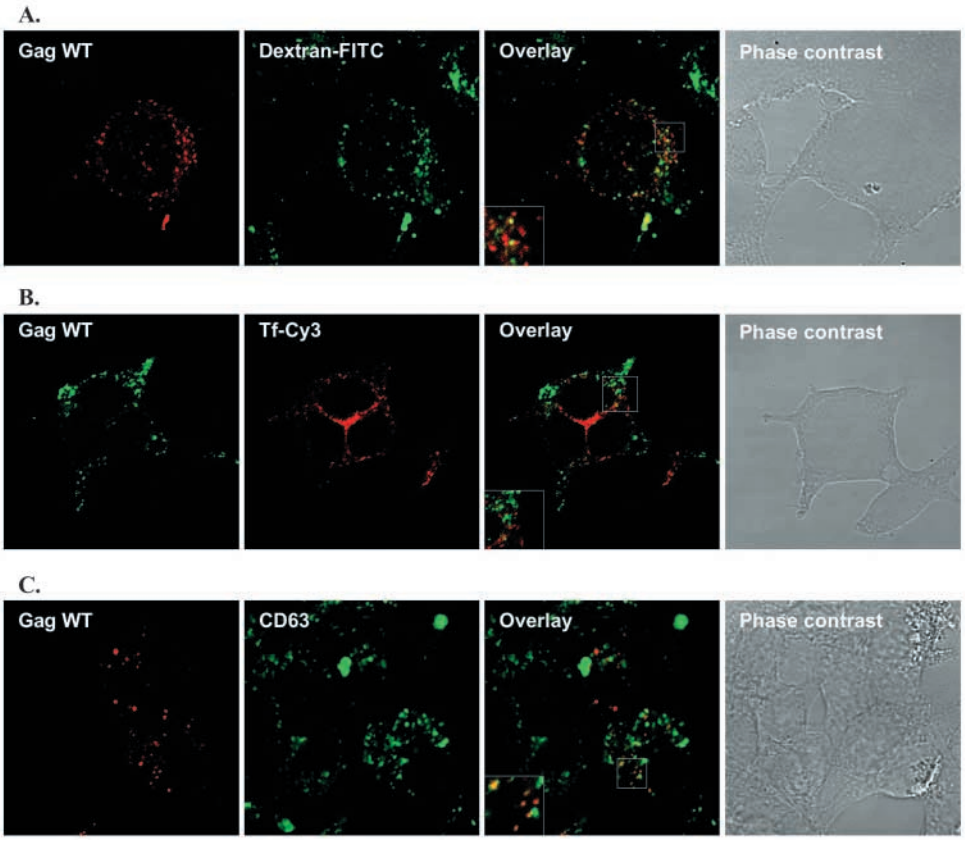

Fig. 4. Wild-type HTLV-1-Gag proteins localize mainly in the endocytic pathway in 293T cells at steady state. (A) Gag co-staining with the whole endocytic pathway revealed by 1-hour uptake of FITC-conjugated dextran. (B) Gag co-staining with early/recycling endosomes revealed by 1-hour uptake of cyanin 3-conjugated transferrin. (C) Gag co-staining with the late endosomes/MVBs marker CD63. Each row shows a median section of cells recorded by confocal microscopy using a 63× objective and a 4× zoom. Overlays of red and green pictures are shown as well as bright light pictures. Zoomed images of colocalization areas are also presented (boxed).

we found that Tsg101 depletion did not affect the localization of the Y121D mutant viruses, which still accumulated at the plasma membrane (Fig. 6C). Altogether, these results provide strong evidence that the functional interaction of Gag with Nedd4.1 at the plasma membrane precedes the Gag/Tsg101 interaction, which occurs later during Gag intracellular trafficking through the endosomal pathway.

The PTAP motif acts later in the budding process than the PPPY motif

We finally used electron microscopy to visualize the site of virus assembly in conditions preventing Gag from interacting with Nedd4.1 or Tsg101. Virus particles were detected in the extracellular medium of cells transfected with the wild-type provirus (Fig. 7A, panels 1 and 2). We also observed a few electron-dense thickenings underneath the plasma membrane (Fig. 7A, panels 1 and 3, arrows). As previously reported (Le Blanc et al., 2002), we found that viruses mutated in the PPPY motif accumulated underneath the plasma membrane and appear as electron-dense thickenings of variable sizes (Fig. 7B, arrows). This peculiar morphology indicates that the virus was blocked at an early stage of the budding process. Viruses mutated in the PTAP motif displayed various morphologies (Fig. 7C, panel 4), although often appearing as immature electron-dense cores still tethered to cellular membranes (Fig. 7C, panels 1-4, arrows). Importantly, PTAP-mutated virions were not seen at the plasma membrane but rather accumulated in the lumen of intracellular vesicles (Fig. 7C, panels 1-4).

These results, which extend our confocal microscopy observations, provide further evidence that the late steps of HTLV-1 release take place in intracellular vesicles, involving the PTAP motif, whereas the PPPY motif acts earlier in the budding process, at the plasma membrane.

Discussion

In this study, we explored the functions of Nedd4 and Tsg101, two cellular trafficking proteins, in retrovirus budding. For this purpose, we studied the budding process of the human oncoretrovirus HTLV-1, the Gag proteins of which harbor tandem PPPY/PTAP motifs.

As L domains that contain determinants for budding can be functionally exchanged between several retroviruses (Accola et al., 2000; Parent et al., 1995; Yuan et al., 2000), it is generally assumed that PPxY and PTAP motifs mediate equivalent, and therefore redundant, functions in retrovirus release (reviewed by Freed, 2002). Our results are not consistent with this model as they reveal that the efficient release of HTLV-1 requires the integrity of both the PPPY and the PTAP motifs, as recently reported (Bouamr et al., 2003). MPMV, another oncoretrovirus, also uses both a PPPY and a PSAP motifs for budding (Gottwein et al., 2003). Furthermore, sequences other than the PTAP motif in HIV Gag p6 protein also account for virus budding (Martin-Serrano and Bieniasz, 2003; Strack et al., 2003). Finally, overlapping PPxY and PTAP motifs in the matrix protein of Ebola virus are also required for the release of virus-like particles (Harty et al., 2000; Licata et al., 2003; Martin-Serrano et al., 2001; Timmins et al., 2003). Hence, the use of two distinct budding motifs might be a general property shared not only by oncoretroviruses and lentiviruses, but also by other RNA-enveloped viruses.

Retroviral Gag PPPY motifs were shown to recruit members of the Nedd4 family of E3 ubiquitin ligases (Kikonyogo et al., 2001; Yasuda et al., 2002), but the importance of the enzymatic activity of these proteins remained to be assessed. We demonstrate here that HTLV-1 budding requires the

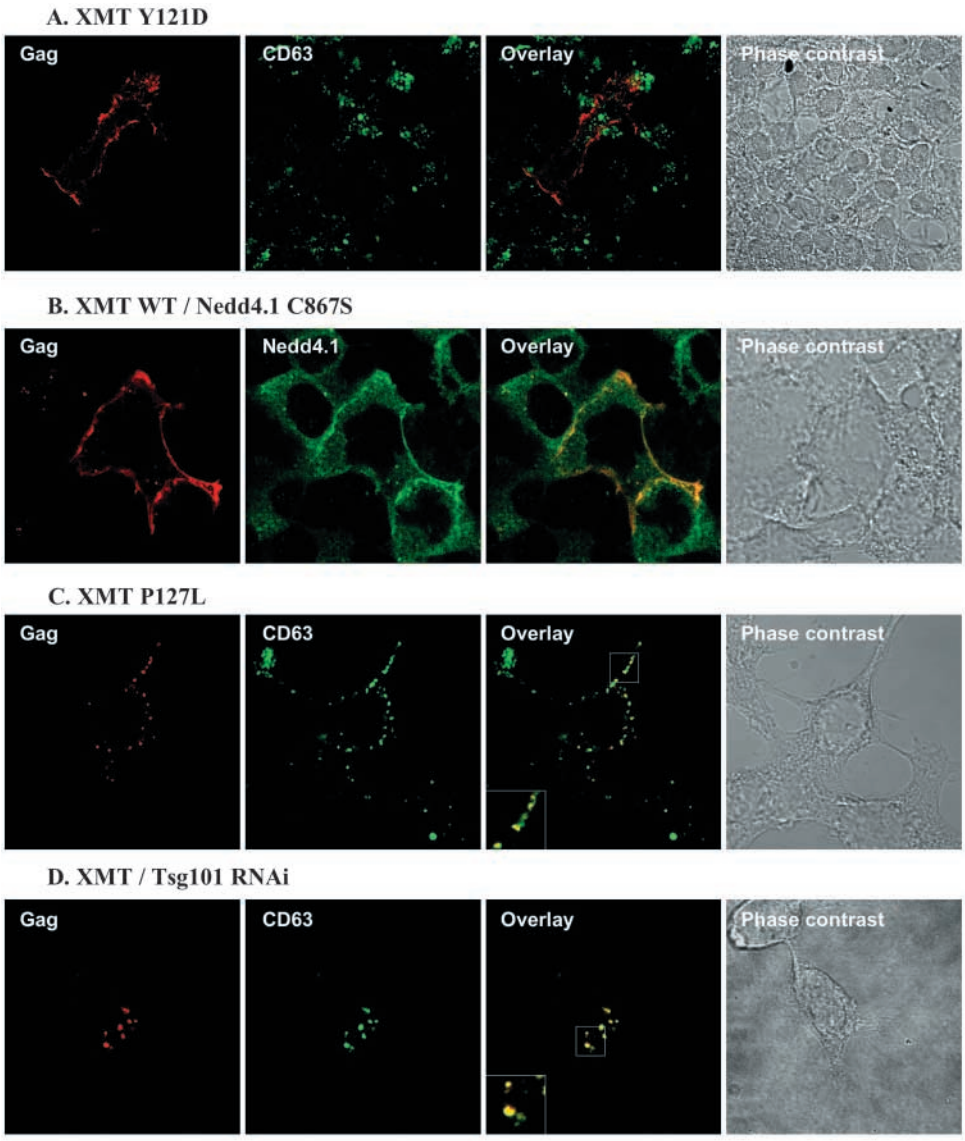

Fig. 5. PPPY/Nedd4.1 and PTAP/Tsg101 interactions take place in different compartments. (A) Gag/CD63 co-staining in 293T cells expressing the Y121D-mutated provirus. (B) Gag/Nedd4.1 co-staining in 293T cells transfected with the wild-type provirus and Nedd4.1-C867S-expressing construct. (C) Gag/CD63 co-staining in 293T cells expressing the P127L-mutated provirus. (D) Gag/CD63 co-staining in 293T cells transfected with the wild-type provirus and Tsg101 siRNAs. Each row shows a median section of cells recorded by confocal microscopy using a 63× objective and a 2× (A) or 4× (B,C,D) zoom. Overlays of red and green pictures are shown as well as bright light pictures. Zoomed images of colocalization areas are also presented (boxed).

recruitment by Gag proteins of isoform 1 of human Nedd4. As expected, Nedd4.1 is recruited via the canonical PPPY motif/WW domain interaction. We also report that overexpression of a dominant-negative, enzymatically inactive mutant of Nedd4.1 strongly interferes with the release of HTLV-1 particles. This effect was specific, as overexpression of a dominant-negative mutant of Nedd4.2 had only a slight effect. Furthermore, we demonstrated that HTLV-1 Gag proteins are ubiquitinated and that this process depends both on the integrity of the PPPY motif and on the presence of functional Nedd4.1, but not Nedd4.2. Hence, the requirement of a specific isoform of Nedd4 for efficient HTLV-1 release correlates directly with its ability to promote Gag ubiquitination. This provides the first direct evidence that the ubiquitin ligase activity of a specific E3, namely Nedd4.1, is required for retroviral release.

Proteasome inhibitors interfere with the particle release process of several enveloped viruses (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). We found that these drugs also induced the intracellular retention of immature or partially mature HTLV-1 Gag (our unpublished results), which is reminiscent of the effect of mutations in Gag PPPY motif (Le Blanc et al., 2002). Defects in retrovirus budding caused by proteasome inhibitors can be overcome either by overexpressing free ubiquitin or by fusing Gag to ubiquitin (Patnaik et al., 2000), which suggests that Gag ubiquitination is important per se for efficient virus release, rather than functional proteasome machinery. The exact role of Gag ubiquitination in virus release is not fully understood. It has been demonstrated that conjugation of mono-ubiquitin or of short ubiquitin chains to the cytoplasmic domains of plasma membrane proteins can trigger their endocytosis (Shih et al., 2000). Ubiquitination of retroviral Gag could also serve as an endocytic signal, as the expression of dominant negative mutants of ubiquitin that specifically inhibit the ubiquitin-mediated endocytosis drastically affects HIV-1 budding (Strack et al., 2002). Our data strongly confirm this mechanism, as we found that HTLV-1 Gag was mainly mono-ubiquitinated and that preventing Gag from interacting with Nedd4.1, which precludes Gag ubiquitination, induces the relocalization of HTLV-1 Gag from endocytic compartments to the plasma membrane. Consistently, electron microscopy experiments showed that viruses mutated in the PPPY motif accumulate underneath the plasma membrane, arrested early in the budding process. The fact that ubiquitination can serve as an endocytic signal for retroviral Gag would also explain why the EIAV lentivirus, the Gag proteins of which directly recruit type-2 clathrin adaptors (AP-2) that permit endocytosis of plasma membrane proteins (Puffer et al., 1998), is insensitive to proteasome inhibitors (Ott et al., 2002; Patnaik et al., 2002). It remains unclear whether Gag proteins that do not interact with

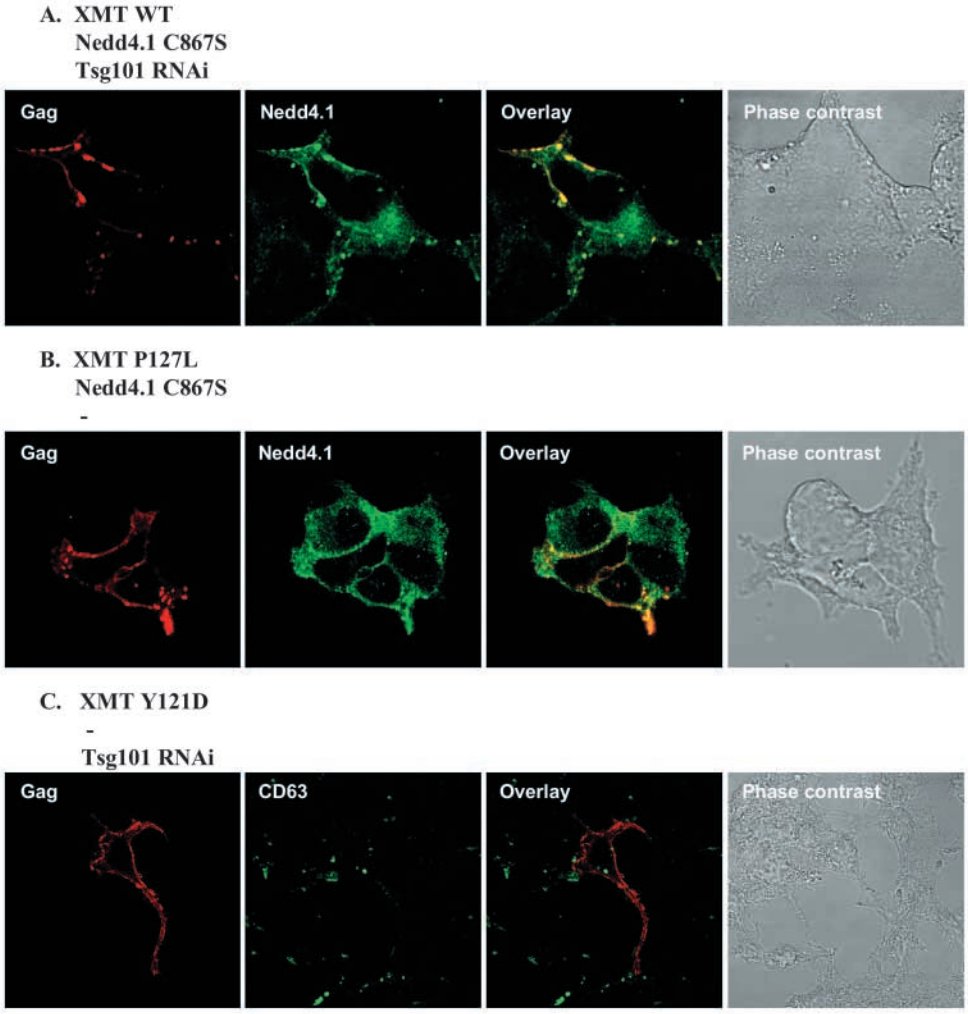

Fig. 6. Gag/Nedd4.1 interaction at the plasma membrane precedes Gag/Tsg101 interaction in late endosomes. (A) Gag/Nedd4.1 co-staining in 293T cells co-transfected with wild-type XMT provirus, Tsg101 siRNAs and Nedd4.1-C867S construct. (B) Gag/Nedd4.1 co-staining in 293T cells co-transfected with XMT P127L provirus and Nedd4.1-C867S construct. (C) Gag/CD63 co-staining in 293T cells co-transfected with XMT Y121D provirus and Tsg101 siRNAs. Each row shows a median section of cells recorded by confocal microscopy using a 63× objective and a 4× (A,C) or 2× (B) zoom. Overlays of red and green pictures are shown as well as bright light pictures.

Nedd4 or AP-2 also recruit a functional endocytic machinery or whether they reach endocytic compartments via alternative mechanisms.

HIV and MPMV PS/TAP motifs are known to recruit Tsg101 (Garrus et al., 2001; Gottwein et al., 2003). By depleting Tsg101 in HTLV-1-producing cells, we demonstrated that this protein is also necessary for HTLV-1 release. Consistently, HTLV-1 Gag is able to associate with Tsg101 in vitro (Bouamr et al., 2003).

Tsg101 is part of a complex of proteins named ESCRT-1, involved together with ESCRT-2 and -3, in the sorting of ubiquitinated proteins into internal vesicles of MVBs (Babst et al., 2002a; Babst et al., 2002b). As noticed previously (Garrus et al., 2001), the formation of internal vesicles of MVBs is topologically similar to the budding of enveloped viruses at the plasma membrane, as in both cases, membranes invaginate away from the cytoplasm towards the lumen. It has thus been proposed that viral Gag proteins can delocalize Tsg101 and the cellular MVB budding machinery to the site of virus budding at the plasma membrane (Martin-Serrano et al., 2001; Martin-Serrano et al., 2003). However, we found that rather than blocking HTLV-1 at the plasma membrane, Tsg101 depletion or mutation of the PTAP motif in Gag results in the retention of Gag proteins in vesicular structures corresponding to late endosomes/MVBs. This strongly supports the view that the functional interaction between HTLV-1 Gag and Tsg101 does not take place at the plasma membrane but rather where ESCRT-1 complex functions, namely the late endosomes. This was also illustrated by the detection of wild-type Gag proteins in the endocytic pathway, especially in CD63-positive compartments. The fact that HTLV-1 Gag proteins were found in the endocytic pathway may appear to contradict a previous report stating that Gag intracellular localization was independent of intracellular membranes (Le Blanc et al., 2002). As the previous study was performed in HeLa cells and this one used 293T cells, the apparent discrepancy in Gag localization may result from cell type differences. Interestingly, we observed that retroviral particle release was significantly less efficient in HeLa cells than in 293T, suggesting that there is a strong relationship between the ability of Gag to traffic through the endocytic pathway and the efficiency of virus particle release. Consistent with our present observations, other retroviral Gag proteins have been found to be associated with endocytic compartments (Basyuk et al., 2003; Goff et al., 2003; Sfakianos and Hunter, 2003). Recent studies also demonstrated that retroviral particles can assemble and bud in intracellular compartments in various cell types (Nydegger et al., 2003; Sherer et al., 2003). The fact that late steps of retrovirus assembly and budding occur in late endocytic compartments implies that additional mechanisms account for subsequent virus release at the plasma membrane. One exciting possibility is that Gag proteins follow the cellular pathway that leads to exosome secretion at the plasma membrane (Thery et al., 2002). In this regard, HIV-1 virions bud preferentially in MVBs in macrophages and are released after fusion of these compartments with the plasma membrane (Pelchen-Matthews et al., 2003; Raposo et al., 2002).

It has been recently suggested that Tsg101 and Nedd4 might

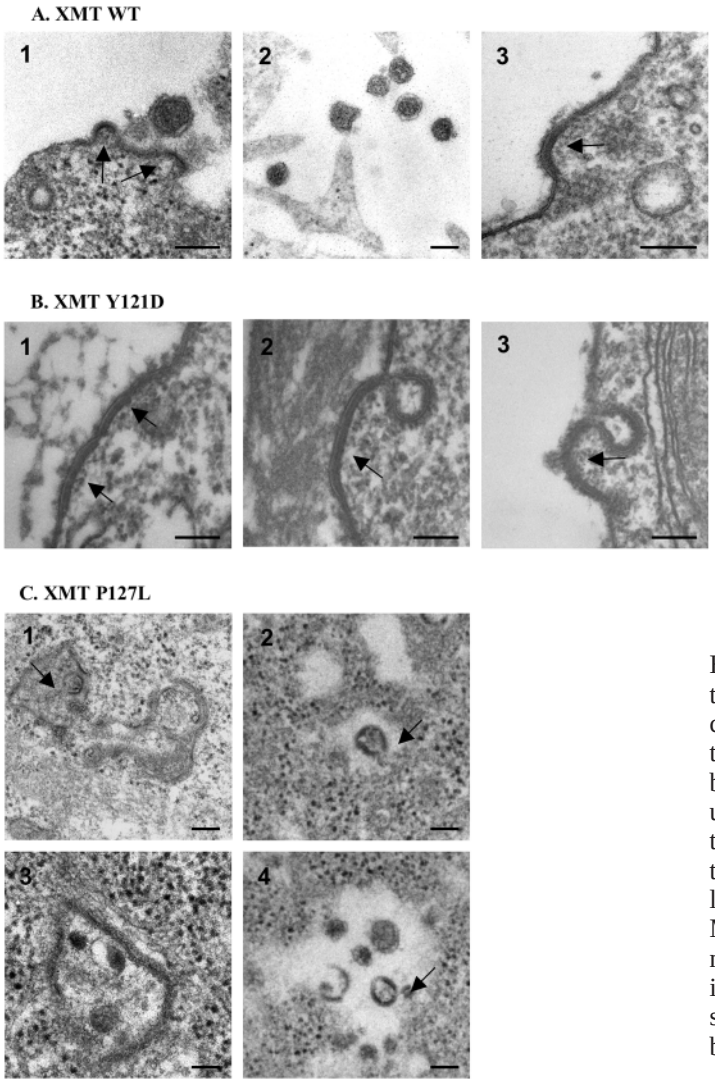

Fig. 7. Wild-type, Y121D and P127L virus particles display distinct cellular localizations and morphologies. (A) Electron micrographs of 293T cells transfected with the wild-type XMT provirus. Arrows point to electron dense thickenings underneath the plasma membrane. Scale bars represent 100 nm. (B) Electron micrographs of 293T cells transfected with the Y121D-mutated provirus. Arrows point to electron dense thickenings underneath the plasma membrane. Scale bars represent 50 nm. (C) Electron micrographs of 293T cells transfected with the P127L-mutated provirus. Arrows point to particles still tethered to cellular membrane. Bars, 100 nm.

respectively act at an early and late stage of HTLV-1 budding (Bouamr et al., 2003). Our data clearly support the opposite model as we obtained strong evidence that HTLV-1 Gag encounters Nedd4.1 before Tsg101. Indeed, preventing Gag from interacting with Tsg101 had no effect on the plasma membrane localization of PPPY-mutated viruses whereas preventing Gag from interacting with Nedd4.1 induced the relocalization of PTAP-mutated Gag from internal vesicles to the plasma membrane. Finally, preventing wild-type Gag from interacting with both Nedd4.1 and Tsg101 also provoked Gag accumulation at the plasma membrane. This last result argues against a model in which Gag molecules interact randomly with one protein or the other at any given time because in this case, Gag proteins would have been distributed both at the plasma membrane and in internal vesicles. Moreover, we found that PTAP-mutated HTLV-1 particles often displayed the characteristic curvature of nearly formed particles, whereas PPPY-mutated viruses appeared as unbent dense materials, blocked therefore at an earlier stage of particle formation. Hence, our results indicate that Gag/Nedd4.1 interaction at the plasma membrane occurs before Gag/Tsg101 interaction in late endosomes. Interestingly, ubiquitination of HIV Gag strongly increases its affinity for Tsg101 (Pornillos et al., 2002a). That Gag proteins interact with Nedd4.1, which promotes Gag ubiquitination, before interacting with Tsg101 may thus ensure efficient Gag recognition by Tsg101. Therefore, our results suggest that HTLV-1 Gag intracellular trafficking mimics this of cellular plasma membrane proteins that are sorted to MVBs after ubiquitin-dependent endocytosis (Katzmann et al., 2001).

In conclusion, we demonstrated that the release of HTLV-1 particles requires the complementary engagement of two budding motifs that act successively by recruiting two cellular proteins of the endocytic pathway, eventually leading to Gag sorting into late endosomes/MVBs. The mechanisms by which internal vesicles of MVBs are formed remain largely unknown. However, there are considerable similarities between the process of vesicle budding inside the lumen of MVBs and the mechanism by which RNA enveloped viruses acquire their lipid bilayer. The identification of new factors involved in the MVB pathway should therefore provide new insights into the mechanism of RNA enveloped virus budding. Conversely, the investigation of the late stages of enveloped virus assembly should help to elucidate these fundamental aspects of cell biology.

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