Tsg101 and Alix Interact with Murine Leukemia Virus Gag and Cooperate with Nedd4 Ubiquitin Ligases during Budding*

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Retroviruses use endosomal machinery to bud out of infected cells, and various Gag proteins recruit this machinery by interacting with either of three cellular factors as follows: ubiquitin ligases of the Nedd4 family, Tsg101, or Alix/Aip1. Here we show that the murine leukemia virus Gag has the unique ability to interact with all three factors. Small interfering RNAs against Tsg101 or Alix and dominant-negative forms of Nedd4 can all reduce production of virus-like particles. However, inactivating the Nedd4-binding site abolishes budding, whereas disrupting Tsg101 or Alix binding has milder effects. Nedd4 ubiquitin ligases are therefore essential, and Tsg101 and Alix play auxiliary roles. Most interestingly, overexpression of Alix can stimulate the release of Gag, and this occurs independently of most Alix partners Tsg101, Cin85, Alg-2, and endophilins. In addition, Gag mutants that do not bind Tsg101 or Alix concentrate on late endosomes and become very sensitive to dominant-negative forms of Nedd4 that do not conjugate ubiquitin. This suggests that the direct interaction of Gag with Tsg101 and Alix favors budding from the plasma membrane and relieves a requirement for ubiquitination by Nedd4.1. Other Nedd4-dependent Gag proteins also contain binding sites for Tsg101 or Alix, suggesting that this could be a common feature of retroviruses.

In the last years, our understanding of the mechanisms underlying retroviral budding has made enormous progress. It is now well appreciated that Gag proteins of many retroviruses hijack the budding machinery of multivesicular bodies (MVB), allowing the nascent viral particle to divert this machinery for its own use (1–8). Indeed, in the cell, MVB have the unique ability to generate intra-luminal vesicles, and this occurs with a topology identical to that of retroviral budding. Sorting of cellular proteins toward internal MVB vesicles (referred to as the MVB pathway), often requires their mono- or di-ubiquitination, and the ubiquitin moiety often constitutes an essential routing signal (9–14). In yeast, the sorting and budding machinery of MVB is composed of more than 15 proteins that can be biochemically separated into three complexes, termed ESCRT-I, -II, and -III (9, 11, 15). ESCRT-I and -II are believed to be involved in the recognition and sorting of ubiquitinated proteins and in the recruitment of ESCRT-III, which likely represents the core machinery effecting the budding and membrane fission events required for vesicle formation. In addition to these well defined complexes, several proteins that are essential for this machinery occur separately or are loosely associated with these complexes. This is the case for the yeast protein Bro1, which interacts with ESCRT-III (16).

Most components of the ESCRT complexes are conserved in vertebrates (3, 6, 10, 14, 17, 18), and retroviral Gag proteins interact directly with some of them (1, 3, 4, 7, 8, 19–30). This probably allows Gag to recruit ESCRT-III and to bud out of infected cells (3, 4, 29). Mutation of Gag in its ESCRT interaction sites causes a late defect in viral budding, in which viral particles remain attached to the cell membrane and are not released (1, 3, 4, 23, 31, 32). Remarkably, despite the large diversity of retroviruses and the more than 20 possible targets on this sorting pathway, only three cellular factors have been found to interact directly with Gag proteins (1, 3, 4, 7, 8, 19–30). The first are the ubiquitin ligases of the Nedd4 family, which are involved in the ubiquitination of membrane-associated proteins to initiate their sorting toward internal MVB vesicles (10, 14, 33). Nedd4 proteins bind short PSEXY motifs on Gag through conserved WW domains, and they have been shown to interact with MLV, RSV, HTLV, and MPMV Gag (19, 21–28, 34). In agreement, a number of Gag proteins become mono- or di-ubiquitinated during the budding process (35–37). The second protein is Tsg101, an essential component of ESCRT-I that has been shown to bind the Gag proteins of HIV-1, HIV-2, MPMV, and HTLV-I (1, 7, 8, 21, 22, 25, 27, 30). In these cases, budding occurs through P(T/S)AP motifs present in Gag, which mimic the natural interaction of Tsg101 with its cellular partner CHMP4 (38). Tsg101 also binds ubiquitin through a UEV domain, and ubiquitination of Gag increases its affinity for Tsg101 (1, 38). Finally, and more recently, Alix has also been shown to be involved directly in HIV-1 and ELAV budding (3, 4, 29). Alix is the homolog of yeast Bro1, an essential protein of the MVB pathway (16). In mammals, it has been shown to interact with both Tsg101 and CHMP4, a core component of ESCRT-III (3, 4, 29), and it binds Gag proteins through LYPX_{3,4}L motifs (3, 4, 29). Gag proteins can also recruit cellular factors involved in endosomal function but not directly linked to the MVB pathway. For instance, MLV Gag has been shown to interact with endophilins (39). HIV-1 Gag also interacts with the clathrin adaptor protein complex 3 (40), and this has been shown to control its intracellular routing.

It is not clear why the viral Gag proteins have selected...
Nedd4, Tsg101, and Alix among all other proteins of the MVB pathway. Even more puzzling, viral Gag proteins often contain several interacting motifs, and in different viruses, their respective importance can vary. For instance, HIV-1 Gag binds Tsg101 and Alix, but Tsg101 is essential, whereas Alix plays only an auxiliary role (1, 3, 4, 29). In contrast, Alix binds to EIAV Gag and is required for its budding (4, 29), whereas Tsg101 binds to MPMV Gag but is not essential (25).

In this work, we have analyzed the budding partners of MLV Gag, and we have found that, most surprisingly, it interacts with Nedd4, Tsg101, and Alix. This is the first Gag protein shown to interact with all three factors, and it gave us a unique opportunity to analyze their respective role in budding.

EXPERIMENTAL PROCEDURES

Plasmids, Mutagenesis, and Two-hybrid Assays—

Monoloy MLV Gag was cloned in the donor vector of the Gateway cloning system (Invitrogen), and mutagenesis was performed on this plasmid with the quick-strand mutagenesis kit (Stratagene). RSV Gag (a gift of Dr. J. L. Darlix, GenBank® accession number M37980) was also cloned in the Gateway system. Wild-type and mutant Gag cDNAs were then cloned into two-hybrid plasmid pACT-II and pAS2-1 plasmids, which encode transcription vectors with and without C-terminal YFP tags. Mouse Alix was also cloned into pACT-II, and two-hybrid vectors for human Tsg101 and rat Nedd4 were gifts from Dr. W. Sundquist and Dr. D. Rotin, respectively (1, 41). The dominant-negative Vps4 mutant was a gift of Dr. Woodman and Dr. Oshami (42, 43). For the ctre-PSAP mutant, the sequence GGFLPSASPpLP was introduced in-frame at the C terminus of the MLV Gag triple mutant that binds neither Tsg101, Alix, nor Nedd4.

Mouse Alix had a FLAG tag (44). AlixΔPGY was deleted for amino acids 802–813; Alix ΔTsg101 was deleted for amino acids 717–720; AlixΔCin85 was deleted for amino acids 739–745; and AlixΔPP14 was deleted for amino acids 748–761. Wild-type Nedd4.1, as well as wild-type Nedd4.2, were re-cloned at the C terminus of YFP, GST-Gag, GST-MAP12, and GST-Alix were generated by cloning corresponding cDNAs at the C terminus of GST, in the pDest27 plasmid (Invitrogen). CFP-TiYamp was cloned with the Gateway technology and was expressed under the control of mouse ribosomal L30 promoter.

For the two-hybrid assays, plasmids were introduced into the appropriate haploid strains (CG292 or YL455), which were then crossed. Diploids were plated on double or triple selectable media (minus Leu, Trp, or minus Leu, Trp, His), and growth was assessed 3 days later.

Analyses of Virus-like Particles (VLPs), Formation and Release—

293T cells cultured in standard conditions were transfected with the calcium-phosphate procedure (10 μg of plasmids for a 60-mm plate), as described (45). After 15 h, cells were harvested, centrifuged, and extracts were incubated with glutathione-Sepharose beads (2 h at 4 °C). The beads were washed four times in PBS/Triton 0.1%, and resuspended in Laemmli buffer. Bound proteins were analyzed by Western blotting. HNTG contained 20 mM Hepes, pH 7.9, 150 mM NaCl, 1% Triton, 10% glycerol, 1 mM MgCl2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Roche Applied Science).

siRNA—

293T cells were transfected with Lipofectamine in 60-mm dishes (11.1 μl of Lipofectamine Plus and 11.1 μl of Plus reagent in a final volume of 2.8 ml of Opti-MEM), using a mixture of a Gag-YFP expression vector (2 μg) and the indicated siRNA (100 nm). Cells were washed in Opti-MEM and incubated with the transfection mixture for 6 h. Complete media without antibiotics were then added, and media were changed 24 h later. Following an additional 24-h period, cells and cell culture supernatants were harvested and analyzed by Western blotting. The siRNA duplexes had two deoxynucleotides of 3′-overhang. They had the following sequence (only the reverse strand is shown): Tsg101, 5′-GAGGAGAAGACUGGGAGGdTdT; Alix, 5′-GAUGAACCACAGCGGdTTdT; Nedd4.1, 5′-AACGACCAGGGCCUCdTdT; and Nedd4.2, 5′-CUCUGACUUGUUGUUUGGdTTdT.

Antibodies—

Anti-Nedd4.2 antibodies were the gifts of O. Staub. Anti-MLV Gag (a gift from Dr. G. Bolognesi) were from Western Blotting, Inc. Anti-Alix antibodies were described previously (44). Anti-Tsg101 antibodies were from Abcam. Anti-FLAG antibodies were from Sigma. Rabbit polyclonal anti-GFP (Molecular Probes) was used to detect YFP.

RESULTS

MLV Gag Interacts with Nedd4, Tsg101, and Alix—

In the budding partners of MLV Gag reveals three potential late motifs (Fig. 1A). One is a PSAP motif found in the matrix (MA); the other is a LYPAL sequence found at the junction between MA and PI2, and the last is a PPYY motif in PI2. The PPYY motif has been shown previously to be important for budding and to interact with a variety of ubiquitin ligases of the Nedd4 family (WWP1, WWP2, and AIP4; see Ref. 46). However, the exact identity of the ligase involved in promoting its budding is still mysterious. To test if these motifs could bind their expected partner in the context of the full-length protein, we cloned MLV Gag in a two-hybrid vector and tested the interactions with Tsg101, Alix, and Nedd4. Remarkably, MLV Gag could indeed interact with these three proteins (Fig. 1B). To test if the interactions occurred at the expected site, each motif was mutated, and the mutant proteins were tested in the two-hybrid assay. As expected, a PSAA mutant in the PSAP motif abolished the interaction with Tsg101, whereas both LYPAL and LYPA mutants in the LYPAL motif prevented interaction with Alix, and a PPYY mutant of the PPYY motif blocked the binding of Nedd4. The effect of these mutations was not due to a global alteration of the protein structure because each mutant abolished only one interaction and was still interacting with the other two proteins.

To show that the two-hybrid results reflected real interactions in mammalian cells, the binding of MLV Gag to Nedd4, Tsg101, and Alix was investigated by co-immunoprecipitation analysis. Mammalian cells express several isoforms belonging to the Nedd4 family and, in particular, the Nedd4.1 and Nedd4.2 isoforms, which can bind overlapping sets of partners in vivo (47). In addition, although MLV Gag was shown to bind WWP1, WWP2, and AIP4 (34), its interactions with Nedd4.1 and Nedd4.2 were not documented. Thus, 293T cells were co-transfected with vectors expressing a GST-Gag fusion and either Nedd4.1 or Nedd4.2 fused to YFP. As control, we used the triple mutant of Gag unable to bind either
Nedd4, Tsg101, or Alix. The proteins bound to GST-Gag were purified on glutathione-Sepharose beads and analyzed by Western blotting with anti-YFP antibodies. Both YFP-Nedd4.1 and YFP-Nedd4.2 co-precipitated with GST-Gag but not with its mutant counterpart (Fig. 2A), indicating that these proteins could indeed bind Gag in vivo. To confirm these results, we repeated the experiment without co-transfecting Nedd4 expression vectors, and we found that MLV Gag also interacted with endogenous Nedd4 proteins (Fig. 2A). Similar experiments revealed that GST-MA-p12 and GST-Gag, but not their mutant versions, could also bind endogenous Tsg101 (Fig. 2B and data not shown). Finally, to document the interaction of Gag with Alix, 293T cells were co-transfected with vectors coding for Gag-YFP and GST-Alix. A fraction of Gag-YFP, but not its mutant version, was found to co-precipitate with GST-Alix, indicating that these proteins interacted in vivo (Fig. 2C). We could not, however, co-immunoprecipitate endogenous Alix with MLV Gag, suggesting that these interactions were too weak and/or too transient to be maintained during the course of the experiment.

Altogether, these results show that MLV Gag has the unique ability to interact with Tsg101, Alix, and Nedd4 ubiquitin ligases. These interactions occur through canonical binding motifs, which are located in the same area of the protein, from the end of MA to the middle of P12.

The PPPY Motif in MLV Gag Is Essential for MLV Egress, whereas the PSAP and LYPAL Motifs Increase Budding Efficiency—To test the role of Nedd4, Tsg101, and Alix on MLV Gag budding, we mutated each of their binding sites on Gag, alone, and in combination, and we analyzed the amount of VLPs produced following transfection in 293T cells. To detect even slight defects in viral production, we collected supernatants two times following transfection, 15 and 38 h. As shown in Fig. 3A, and as described previously (34, 46, 48), the PPPA mutant in the PPPY motif was sufficient to induce a drastic reduction in the number of VLPs produced. Most interestingly, the mutation of the Tsg101- and Alix-binding sites (PSAA and LYPAA, respectively) also led to a decrease in the number of VLPs produced. However, this effect was limited because the decrease was mild and could be detected only at the earliest 15-h time point. The second mutation of the Alix-binding site (LAPAL) had little or no effect, suggesting that this mutation was less detrimental in vivo. To extend these results, we performed a quantitative Western blot assay at the 15-h time point (see “Experimental Procedures”). We found that the amount of VLPs produced by the PSAA (Tsg101) and LYPAA (Alix) mutants was 35% that of wild-type Gag, whereas the double mutant PSAA/LYPAA showed a stronger defect (only 20% of wild-type Gag). In contrast, the PPPA (Nedd4) mutant inhibited VLP production to more than 95%, both at the 15- and 38-h time points. Altogether, these results suggested that binding of Nedd4 ubiquitin ligases was essential for
only 10% and 1% of the wild-type VLPs were loaded (lanes WT-10% and WT-1%, respectively).

The indicated constructs were transiently transfected in 293T cells, and budding of Gag-YFP is inhibited by the mutation of its Tsg101- and Alix-binding sites. Cells were treated and analyzed as in A. D, PSAP motif introduced at the C terminus of MLV Gag partially restores mutation of the PPPY motif. The indicated constructs were transiently transfected in 293T cells, and the amount of cellular and VLPs Gag was determined by Western blotting 38 h following transfection. Lane WT, wild-type Gag; lane 2+3, LYPAA and PPPA double mutant; lane 1+2+3, LYPAA and PPPA double mutant; lane 1+2+3, PSAA, LYPAA, and PPPA triple mutant. B, Gag-YFP buds less efficiently than Gag. Cells were treated as in A, and VLP production was analyzed 38 h after transfection. C, budding of Gag-YFP is inhibited by the mutation of its Tsg101- and Alix-binding sites. Cells were treated and analyzed as in A. D, PSAP motif introduced at the C terminus of MLV Gag partially restores mutation of the PPPY motif. The indicated constructs were transiently transfected in 293T cells, and the amount of cellular and VLPs Gag was determined by Western blotting 38 h following transfection. Lane WT, wild-type Gag; lane 2+3, LYPAA and PPPA double mutant; lane 1+2+3, PSAA, LYPAA, and PPPA triple mutant; lane Cter-PSAP, the Gag triple mutant in which a PSAP sequence was introduced at its C terminus. In the VLP panel, only 10% and 1% of the wild-type VLPs were loaded (lanes WT-10% and WT-1%, respectively).

MLV budding and could not be replaced by an interaction with either Tsg101 or Alix. However, binding of these factors was required for an optimal budding efficiency.

To reveal more clearly the role of Tsg101 and Alix in MLV budding, we tested the same mutants in a second assay, in which the various Gag proteins were fused to YFP. Indeed, such Gag fusions do bud out of cells, but less efficiently that their unfused counterpart (about three times less VLPs are produced, see Fig. 3B), and we observed that they are more sensitive to defects in the budding process (Fig. 3C). For instance, although the PPPA mutant still abolished the release of VLPs (to more than 99%), the PSAA mutant unable to bind Tsg101 now showed a pronounced defect with a 90% reduction of VLPs at 38 h (Fig. 3C). Similarly, the two mutants defective in Alix binding also showed a stronger reduction in production of VLPs. At 38 h, we observed a reduction of 80% and 60% of the VLPs produced for the LYPAA and LAPAL mutants, respectively (Fig. 3C). Altogether, these results confirmed that the Nedd4-binding site was essential for MLV Gag budding and that the Tsg101- and Alix-binding sites contributed to the optimal efficiency of this process.

It is striking that in the case of HIV-1, the Tsg101-binding site is essential to promote budding (1,31), whereas in the case of MLV, it plays only an auxiliary role and is not sufficient to induce budding. However, in these two cases, the Tsg101-binding site does not occur at the same location. It is in MA for MLV, and near the C terminus of Gag in the case of HIV. To test whether this is important, we took the triple mutant of MLV Gag, which binds neither Nedd4, Alix, nor Tsg101, and re-introduced its Tsg101-binding site at the C terminus of the protein. Significant amounts of VLPs were produced in this case (Fig. 3D), although the overall efficiency was much less than for the wild-type Gag (about 30-fold less, see Fig. 3D). This indicated that the location of the late motifs within Gag influenced their ability to promote budding but that it was clearly not the sole determinant of their activity.

**Budding of a Gag-YFP Fusion Is Reduced by the Depletion of Tsg101 and Alix**—The reduction of MLV budding by the mutation of the binding sites for Tsg101, Alix, and Nedd4 implied that these proteins were required in this process. To confirm this possibility, we depleted these factors with siRNAs, and we analyzed production of Gag-YFP VLPs, because this version of Gag is more sensitive to defects in the budding process. 293T cells were co-transfected with a Gag-YFP expression vector and various siRNAs, including a control one that targeted firefly luciferase mRNA. Cell culture supernatants were collected 2 days after transfection, and VLP production was analyzed as above (Fig. 4A). In parallel, the cellular content was also analyzed by Western blotting, both to normalize the transcription efficiency (Fig. 4A) and to control the depletion of the targeted proteins (Fig. 4B). As suggested by the mutational analysis of Gag, the depletion of Tsg101 led to a pronounced reduction in the production of Gag-YFP VLPs (Fig. 4A, only 25% of the control VLPs, ±16%). The depletion of Alix had milder effects, but it also reduced the amount of Gag-YFP VLPs (Fig. 4A, 50% of the control VLPs, ±50%). To control for nonspecific effects of the siRNAs, we repeated this experiment using a version of Gag not fused to YFP. Indeed, budding defects of this Gag are not detected when its interaction sites with Tsg101 or Alix are inactivated and when VLPs are analyzed at late time points after transfection (as done in the siRNA experiments). We observed that Tsg101 or Alix siRNAs were both unable to inhibit Gag budding (Fig. 4C), indicating that the effects seen on Gag-YFP were specific and unlikely to result from a side effect of the siRNAs. Previous studies (1,49) have also shown that budding of MLV Gag is rather insensitive to Tsg101 depletion. However, a mild inhibition was observed at early time points (24 h post-transfection; see Ref. 1), whereas no effects were seen at later time points (48 h; see Ref. 49). This kinetic effect is similar to the results that we have obtained with the Tsg101 mutant Gag proteins, where a transient defect in budding is detected (i.e. at 15 h but not at 38 h, see Fig. 3). Taken together, these data confirmed that Tsg101 and Alix were involved in the budding of MLV Gag.

Most surprisingly, we failed to detect a significant budding defect of Gag-YFP upon depletion of either Nedd4.1 or Nedd4.2. This could be due to several reasons. First, the depletion of these proteins is not complete (Fig. 4B), and the remaining protein levels could be sufficient to achieve efficient VLP production. Second, because Gag interacts with several members of the Nedd4 family in vivo (Fig. 2) (34), the lack of effect of the siRNAs could be due to some redundancy between the various Nedd4 isoforms.

**Gag Mutants Unable to Bind Tsg101 and Alix Are Very Sensitive to Dominant-negative Forms of Nedd4.1**—To document the role of Nedd4 ubiquitin ligases in MLV budding, we turned to strategies that used dominant-negative mutants of these enzymes. For this purpose, we used a point mutant of Nedd4.1 that was unable to conjugate ubiquitin. The effect of the dominant-negative Nedd4.1 was tested on both wild-type Gag and on the PSAA/LYPAA double mutant that does not bind Tsg101 nor Alix. 293T cells were co-transfected with equal
from MVB (50–52). Similarly, in 293T cells, a large fraction of Gag was released outside the cells when Alix was overexpressed, indicating that it was an important effector of MLV budding. Alix contains a number of short motifs, including the TI-VAMP binding motif, the Cin85 binding motif, the Alg-2 binding motif, and the Tsg101 binding motif (54). To explore further the role of Alix in MLV budding, we tested the effect of co-expressing large amounts of Alix with Gag. 293T cells were co-transfected with vectors expressing wild-type Gag (Fig. 4A and B), or its mutant unable to bind Tsg101 and Alix (PSAA+LYPAA, lower panels), and either with wild-type Nedd4.1 (lane 4.1) or with its dominant-negative version that cannot conjugate ubiquitin (lane 4.1D.N). Cells and VLPs were analyzed 38 h after transfection.

In the case of HIV-1, the interaction with Tsg101 plays an essential role in budding, whereas the binding of Alix plays an auxiliary role (3, 4). However, it is possible to increase the efficiency of Gag release by overexpressing Alix (3, 4). To explore further the role of Alix in MLV budding, we tested the effect of co-expressing large amounts of Alix with Gag. 293T cells were co-transfected with both Gag and either a control or an Alix expression vectors (1 and 9 μg of plasmid, respectively), and the amount of VLPs produced was then analyzed (Fig. 7A). Remarkably, much more Gag was released outside the cells when Alix was overexpressed, indicating that it was an important effector of MLV budding.

In vertebrates, Alix has been shown to have several binding partners, but it is not known whether these proteins are involved in retroviral budding. Alix contains a number of short motifs, including the TI-VAMP binding motif, the Cin85 binding motif, the Alg-2 binding motif, and the Tsg101 binding motif (54). To explore further the role of Alix in MLV budding, we tested the effect of co-expressing large amounts of Alix with Gag. 293T cells were co-transfected with both Gag and either a control or an Alix expression vectors (1 and 9 μg of plasmid, respectively), and the amount of VLPs produced was then analyzed (Fig. 7A). Remarkably, much more Gag was released outside the cells when Alix was overexpressed, indicating that it was an important effector of MLV budding.
motifs that can bind endophilins, Cin85, Alg-2, and the ESCRT-I factor Tsg101 (3, 44, 54, 55). In addition, it also binds CHMP4A, a core component of ESCRT-III (3, 4, 29), although the domains of Alix involved in CHMP4A binding are not known in detail. Endophilins and Cin85 are involved in endocytosis, whereas the role of Alg-2 is not understood, although it has been implicated in apoptosis (56). Alg-2 is especially interesting because its interaction with Alix has been shown to be calcium-dependent, suggesting that it may somehow regulate the function of Alix (55, 57, 58). To test a possible connection of Alg-2 with the MVB sorting pathway, we first analyzed its localization in cells expressing a dominant-negative version of the Vps4 ATPase. This mutant blocks the recycling of ESCRT components and provokes the formation of an aberrant endosomal compartment that accumulates many proteins participating in the formation of internal MVB vesicles, including Vps4 itself (42, 43). For this purpose, a Myc-tagged Alg-2 expression plasmid was co-transfected with wild-type Vps4 or its dominant-negative form, and the localization of the protein was analyzed by immunofluorescence in 293T cells (Fig. 7B). In cells that expressed wild-type Vps4, Alg-2 was diffusely localized in the cytoplasm. In contrast, in cells that expressed the mutant Vps4, Alg-2 could be detected on aberrant endosomes, suggesting that its function could be connected to the MVB pathway.

To test the role of Alix partners in MLV budding, we used Alix mutants unable to bind either Alg-2 (55), Tsg101 (3), endophilins (44), or Cin85 (54). We co-transfected these mutants with Gag, and we analyzed the amount of VLP produced (Fig. 7A). Wild-type and mutant Alix proteins could all stimulate the release of Gag. When the amount of VLPs was compared with the amount of Alix present in the cell, the stimulation mediated by AlixΔCin85 was similar to the one obtained with wild-type Alix. In contrast, AlixΔPP14 appeared to stimulate budding more efficiently than wild-type Alix (2.5 times more VLPs were obtained with less Alix expressed), whereas AlixΔTsg101 and AlixΔPGY appeared slightly less active than wild-type Alix (similar amount of VLPs were obtained with more Alix protein). Altogether, these results suggested that the interactions of Alix with Tsg101, Cin85, endophilins, and Alg-2 were not essential to increase the efficiency of Gag budding.

**DISCUSSION**

In this report, we demonstrate that MLV Gag has the unique characteristic of having three late motifs, PSAP, LYPAL, and PPPY, which mediate the binding of three types of factors, Tsg101, Alix, and ubiquitin ligation of the Nedd4 family. With a combination of approaches that used point mutants of Gag, siRNAs against Tsg101 and Alix, and a dominant-negative form of Nedd4, we show that these three factors are involved in MLV budding. Remarkably however, a point mutation in Gag that disrupts Nedd4 binding is sufficient to abolish the production of VLPs, indicating that the binding sites for Tsg101 and Alix are not sufficient to promote MLV egress. These two
proteins appear to rather facilitate budding because the disruption of their interactions with Gag decreases the rate of VLP release. These effects are especially pronounced when Gag is fused to YFP, but they are also observed with unfused Gag, although only at early time points after transfection. In summary, Nedd4 ubiquitin ligases have a central role in MLV budding, whereas Tsg101 and Alix have auxiliary roles. Most interestingly, we have observed that the overexpression of Alix can markedly stimulate the budding of MLV Gag. This gave us the opportunity to analyze more precisely the role of Alix. Indeed, Alix interacts with a number of cellular proteins, and the role of these factors in viral budding is not known. For instance, Alix binds endophilins (44), and their ability to curve membranes could be involved in budding (39, 59). Similarly, Alix interacts with Tsg101 (3, 4, 29), and given the known roles of Tsg101 in HIV-1 budding, this interaction could potentially stimulate the production of MLV virions. Alix also binds Cin85 (54), which is involved in endocytosis and down-regulation of membrane receptors (60, 61). Cin85 is a scaffolding protein that has multiple partners, and it is able to induce their clustering in the cell cytoplasm (62). Thus, Cin85 could play a role in MLV budding, for instance by allowing Alix to direct Gag to proper intracellular sites and by facilitating its interaction with other proteins. Finally, Alix also binds Alg-2 in a calcium-dependent manner (57, 58), and this could potentially regulate Alix function in budding. However, despite all these possibilities, we found that Alix mutants unable to bind either Cin85, Tsg101, Alg-2, or the endophilins are still able to stimulate the release of MLV Gag. These results indicate that the stimulatory effect of Alix is independent of the partners we have tested. Most interestingly, the Alix mutant that does not bind endophilins is significantly more efficient in the stimulation of budding, suggesting that endophilins may antagonize this activity of Alix. It is currently unclear how Alix stimulates budding of MLV Gag. The mechanisms could be indirect, as suggested by our observation that binding of Alix to Gag is rather weak (we failed to detect co-immunoprecipitation of Gag with endogenous Alix). They could also depend on the direct interaction of Gag with Alix that we could detect upon its overexpression. One interesting possibility is that Alix would bind Gag and, by virtue of its interaction with CHMP4 proteins (3), would then allow recruitment of ESCRT-III. Despite the small effects of the mutations of the binding sites for Tsg101 and Alix on VLP production, these sites are extremely well conserved among various MLV strains (data not shown). Even more strikingly, examination of the sequences of other retroviruses revealed that RSV Gag had also potential binding sites for both Tsg101 and Alix (ASAP and LYPSL at amino acids 168–171 and 180–184, respectively), in addition to the well characterized and essential Nedd4-binding site (PPFY at amino acids 171–174; Fig. 8A) (28). Furthermore, similar to MLV, these motifs are in close proximity to each other near the MA region. To document these potential interactions, we performed a two-hybrid analysis with full-length RSV Gag, and indeed, we observed that it could interact with Tsg101 and Alix in this assay (Fig. 8B). Thus, the existence of binding sites for Tsg101 and Alix in distant MLV isolates and other Nedd4-dependent retroviruses suggests that these sites are important. Two results hint at the functions of Tsg101 and Alix in MLV budding. First, although wild-type MLV Gag is present both at the plasma membrane and in endosomes, mutations of the binding sites for Tsg101 and/or Alix markedly enhance the endosomal localization of Gag. A similar phenomenon has been observed previously for HTLV-I Gag; it interacts with both Nedd4.1 and Tsg101, and mutants that do not bind Tsg101 are enriched on late endosomes (21). Second, the
an MLV Gag mutant that no longer binds Nedd4, Tsg101, and Alix is sufficient to partially restore budding (Fig. 3C). The level of budding obtained is, however, quite low (30 times lower than that of wild-type Gag), indicating that the location of the Tsg101-binding site is not the sole determinant of its activity. These results are consistent with previous studies that have swapped late domains between retroviral Gag proteins and that have observed that both the sequence and the location of the late domains are important (49). One possibility to explain the relative inability of MLV and RSV PSAP/ASAP motifs in promoting budding is that the context of these motifs reduces the affinity for Tsg101. In fact, it has been shown that Tsg101 binds ASAP less well than PTAP (1).

Finally, it is interesting to note that certain endogenous retroviruses competent for retro-transposition do not seem to contain any late domains (65). Most interestingly, these particles are usually assembled on the endoplasmic reticulum, and a late budding phenotype, in which the particle would remain attached to the limiting membrane, could facilitate their re-import in the nucleus. Indeed, membranes of the endoplasmic reticulum and the nuclear envelope are mixed during mitosis, and cytosolic particles attached to the endoplasmic reticulum could then become relocated to the nucloplasmic side of the nuclear envelope. In contrast, particles that have completely budded within the endoplasmic reticulum would have to fuse with the internal membrane of the nuclear envelope to access the nucloplasm.

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