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Yoshiko Usami
University of Massachusetts Medical School

Et al.

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Efficient and Specific Rescue of Human Immunodeficiency Virus Type 1 Budding Defects by a Nedd4-Like Ubiquitin Ligase[▽]

Yoshiko Usami, Sergei Popov, Elena Popova, and Heinrich G. Göttlinger*

Program in Gene Function and Expression, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

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To exit infected cells, human immunodeficiency virus type 1 (HIV-1) exploits the vacuolar protein-sorting pathway by engaging Tsg101 and ALIX through PTAP and LYPx$_n$L late assembly (L) domains. In contrast, less-complex retroviruses often use PPxY L domains to recruit Nedd4 family ubiquitin ligases. Although HIV-1 Gag lacks PPxY motifs, we now show that the budding of various HIV-1 L-domain mutants is dramatically enhanced by ectopic Nedd4-2s, a native isoform with a truncated C2 domain. The effect of Nedd4-2s on HIV-1 budding required a catalytically active HECT domain and was specific, since other Nedd4 family proteins showed little activity and an unrelated retrovirus was not rescued. The residual C2 domain of Nedd4-2s was critical for the enhancement of HIV-1 budding and for the association of Nedd4-2s with Gag, as reflected by its incorporation into virus-like particles. Interestingly, the incorporation of Nedd4-2s also depended on its active site, indicating that the ability to form a thioester with ubiquitin was required. These data suggest a novel mechanism by which HIV-1 Gag can connect to cellular budding machinery.

Both the sorting of ubiquitinated cargo into multivesicular bodies (MVBs) and the biogenesis of these endocytic compartments depends on a network of class E vacuolar-protein-sorting (VPS) proteins (2, 21, 39). MVBs are formed through the inward budding of vesicles from the endosomal membrane, which exhibits the same topology as retroviral budding from the plasma membrane. Indeed, retroviruses exploit the class E VPS protein network to promote their egress from host cells (4, 9, 32).

Most of the class E VPS proteins participate in the formation of three distinct heteromeric endosomal sorting complexes required for transport, known as ESCRT-I, -II, and -III (2). These complexes are transiently recruited to or assemble on endosomal membranes during cargo sorting and appear to function in a sequential manner (2). Membrane-associated ESCRT-III components are recognized by the ATPase Vps4, which catalyzes the disassembly of ESCRT-III and may thereby trigger membrane fission (3, 45). Dominant-negative mutants of Vps4 and of various ESCRT-III components strongly inhibit the release of human immunodeficiency virus type 1 (HIV-1) and of other retroviruses, indicating that a functional MVB pathway is required for efficient retroviral budding (13, 31, 43, 49).

Retroviruses access the class E VPS pathway through so-called late assembly (L) domains in Gag, the viral polyprotein that drives viral particle assembly (4). The primary L domain of HIV-1 consists of a highly conserved PT/SAP motif in the C-terminal p6 domain of Gag (20) and engages ESCRT-I by binding to its component Tsg101 (13, 48). Additionally, HIV-1 p6 harbors a binding site for ALIX/AIP1, another class E Vps protein that also interacts with Tsg101 (43, 49). ALIX binds to L domains of the LYPx$_n$L type and is required for the release of equine infectious anemia virus, a nonprimate lentivirus (12, 31, 43). In the case of HIV-1, the ALIX binding site in p6 provides an auxiliary L domain that is less critical than the PTAP-type L domain (12, 43). However, we recently observed that ALIX also interacts with the nucleocapsid (NC) domain of HIV-1 Gag, which may contribute to HIV-1 release (36).

Retroviruses that do not belong to the lentivirus genus typically use PPxY-type L domains, which also depend on an intact MVB pathway for function (4, 9, 29, 32). When appended to model Gag constructs, PPxY-type L domains induced the formation of Gag-ubiquitin conjugates, indicating that PPxY-type L domains recruit ubiquitin ligase activity (42). The PPxY motif matches the consensus sequence for ligands recognized by WW domains, which are present in E3 ubiquitin ligases of the Nedd4 family (22). This family has nine members in humans and shares a common domain organization, with an N-terminal C2 domain followed by multiple WW domains and a HECT domain. Several lines of evidence indicate that Nedd4 family proteins function in PPxY-mediated budding (6, 17, 27, 53), including the observation that the overexpression of the Nedd4-like protein WWP1, WWP2, or Itch "rescued" release defects caused by PPxY-motif mutations (30). The ability of these E3 ligases to rescue budding depends on the active-site cysteine in the HECT domain, indicating that the ubiquitination of some substrate is required (30). However, how Nedd4 family ubiquitin ligases connect PPxY-type L domains to the MVB pathway is not yet understood.

The release defect of HIV-1 PTAP L-domain mutants can be rescued by increasing the cellular levels of ALIX, and the Tsg101 binding site of ALIX is dispensable for this effect (12, 47). At least in principle, HIV-1 can thus use cellular factors other than Tsg101 for virus release, which may provide an advantage in cells that express only low levels of Tsg101. However, HIV-1 was not expected to be able to use Nedd4 ubiq-

* Corresponding author. Mailing address: UMass Medical School, LRB 526, 364 Plantation Street, Worcester, MA 01605. Phone: (508) 856-2843. Fax: (508) 856-4650. E-mail: heinrich.gottlinger@umassmed.edu.

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uitin ligases instead of Tsg101, since HIV-1 Gag lacks PPxY-type L domains. In spite of this, we show in the present study that the overexpression of Nedd4-2s but not of other Nedd4 family members potently corrects HIV-1 release defects. Nedd4-2s is a native isoform of Nedd4-2 that lacks an intact C2 domain due to alternative exon usage (23). Surprisingly, the WW domains of Nedd4-2s were not involved in the rescue of HIV-1 budding. In contrast, the severely truncated C2 domain of Nedd4-2s was specifically required for the enhancement of HIV-1 budding and for the incorporation of the E3 ligase into virus-like particles (VLP). Interestingly, the uptake of Nedd4-2s into VLP also depended on the active-site cysteine of the HECT domain, indicating that the formation of a thioester with ubiquitin was required.

MATERIALS AND METHODS

Proviral constructs. HXBH10 is a *vpu*-positive version of the infectious HXB2 proviral clone. $HXBH10_{\Delta PTAPP}$ is a variant with an in-frame deletion of codons 7 through 11 of p6 (1). The Δp6 mutant of HXBH10, formerly called the L1/s mutant (14), harbors a premature termination codon in place of the first codon for p6. The Δp6 truncation was also introduced into the p6-coding region of a protease-defective version of the Δ8-87/Δ126-277 minimal Gag construct (5). $Z_{IL}p1p6_{\Delta PTAPP}$ is a protease-positive variant of the previously described $Z_{IL}p6$ Gag construct (1) and was obtained by inserting the ΔPTAPP mutation and the coding sequence for the p1 spacer peptide of HIV-1 Gag into $Z_{IL}p6$. The resulting construct is identical to $HXBH10_{\Delta PTAPP}$ except that the NC coding region is replaced by a sequence providing a heterologous oligomerization domain. The Z_{WT} variant of HXBH10 encodes a chimeric Gag precursor that has NCp1p6 replaced by a leucine zipper dimerization domain (1). The pMPMV proviral plasmid is a cytomegalovirus promoter-driven expression construct for wild-type (WT) Mason-Pfizer monkey virus (MPMV), pMPMV/PY(−) has the PPPY L domain changed to PGAA, and pMPMV/2x(−) additionally has the PSAP L domain changed to AGAP (15).

Expression vectors. Two DNA fragments which together contained the entire coding sequence for the "ancestral" isoform II of Nedd4-2 (11) with an intact N-terminal C2 domain preceded by a FLAG tag were amplified from BC032597 and BC000621 (Open Biosystems), ligated at a common XhoI site, and inserted between the NotI and EcoRI sites of the mammalian expression vector pBJ5. The coding sequences for native Nedd4-2s (residues 122 to 955 of Nedd4-2 isoform II) and for the Δ1-31, Δ1-67, Δ1-103, Δ1-275, or Δ1-432 truncation mutant of Nedd4-2s (residues 153 to 955, 189 to 955, 225 to 955, 397 to 955, or 553 to 955 of Nedd4-2 isoform II) were amplified from BC000621 with or without an N-terminal FLAG tag and inserted into pBJ5. Point mutants of FLAG-Nedd4-2 and FLAG-Nedd4-2s were made using the QuickChange mutagenesis strategy (Stratagene). Expression vectors for WT human Nedd4-1, WWP1, WWP2, and Itch/AIP4 have been described (42) and were used as templates to amplify fragments encoding Nedd4-1s (residues 120 to 900 of Nedd4-1) and WWP1s (residues 110 to 922 of WWP1) with an N-terminal FLAG epitope for insertion into pBJ5. The expression vector for monomeric hemagglutinin (HA)-tagged ubiquitin has also been described (44).

Assays for viral particle production and infectious virus release. 293T cells (1.2×10^6) were seeded into T25 flasks and were transfected 24 h later using a calcium phosphate precipitation technique. The cultures were transfected with HIV-1 proviral DNA (between 0.5 and 1.5 μg) together with expression vectors for WT or mutant Nedd4 family proteins (between 1 and 2.5 μg) and where appropriate for CHMP31-150FLAG (0.5 μg), HA-ubiquitin (1 μg), or empty vector. WT and mutant versions of pMPMV were transfected at 2.5 μg together with 2 μg of empty pBJ5 or Nedd4-2s expression vector. The total amount of transfected DNA was brought to 8 μg with carrier DNA (pTZ18U). Culture supernatants were harvested 24 h posttransfection and clarified by low-speed centrifugation and passage through 0.45-μm filters. Virions or VLP released into the medium were then pelleted through 20%-sucrose cushions. Pelletable material and the cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting as described elsewhere (1), using the anti-HIV CA antibody 183-H12-5C (8) to detect HIV-1 Gag proteins or a goat anti-p27 antiserum to detect MPMV Gag. Epitope-tagged Nedd4 family proteins were detected with anti-FLAG M2 (Sigma), and HA-ubiquitin conjugates were detected with the anti-HA antibody HA.11 (Covance). Infectious titers of HIV-1 were determined in triplicate by challenging TZM-bl indicator cells (1×10^5) seeded into T25 flasks with 1 ml (each) of undiluted 293T-derived supernatant. Saquinavir (2 μM) was added to the target cells to limit infections to a single cycle. Two days postinfection, the indicator cells were lysed in 400 μl reporter lysis buffer supplied with a β-galactosidase (β-Gal) enzyme assay system (Promega), and β-Gal activity induced by HIV-1 Tat as a consequence of infection was measured according to the manufacturer's instructions.

RESULTS

Rescue of a Tsg101-binding-site mutant by an isoform of Nedd4-2. Although HIV-1 Gag lacks PPxY motifs, interactions between Nedd4 and its substrates do not always involve PPxY-mediated mechanisms (33, 51). Since the overexpression of certain Nedd4 family ubiquitin ligases can rescue viral budding in the presence of an attenuated PPxY-type L domain (30), we examined whether members of this family can enhance the budding of HIV-1 L-domain mutants. However, we found no evidence that the overexpression of Nedd4-1, WWP1, WWP2, or Itch/AIP4 significantly affects the release of $HXBH10_{\Delta PTAPP}$ (data not shown).

Nedd4-2 (also called Nedd4L) is a homolog of Nedd4-1 that is expressed either with or without an N-terminal C2 domain (Fig. 1A) as a consequence of alternate proximal exon usage (11, 23, 46). Indeed, the majority of cellular transcripts encoding human Nedd4-2 are predicted to yield a protein, here called Nedd4-2s, that lacks most of the C2 domain (11). Remarkably, when 293T cells were cotransfected with $HXBH10_{\Delta PTAPP}$ and a vector expressing Nedd4-2s, we reproducibly observed a profound increase in viral particle production (Fig. 1A and 2A). Furthermore, Nedd4-2s corrected the Gag processing defect of the ΔPTAPP mutant, which leads to the intracellular accumulation of the Gag cleavage intermediates capsid (CA)-p2 (CAp2) and p41. The ability of Nedd4-2s to alleviate this characteristic cleavage defect of HIV-1 L-domain mutants was highly reproducible and clearly more robust than what we had previously observed for ALIX (47).

Because Nedd4-2s rescued HIV-1 budding in the absence of the primary L domain, we considered the possibility that Nedd4-2s acted independently of the MVB pathway. However, exogenous Nedd4-2s did not suppress the inhibition of HIV-1 budding by $CHMP3_{1-150}FLAG$, a dominant-negative mutant of the ESCRT-III component CHMP3 (54) (Fig. 1B). Furthermore, $CHMP3_{1-150}FLAG$ blocked the rescue of the ΔPTAPP mutant by Nedd4-2s, both in terms of particle production and Gag processing (data not shown). Thus, the overexpression of Nedd4-2s did not circumvent the requirement for an intact MVB pathway.

The ability of Nedd4-like ubiquitin ligases to promote PPXY-dependent budding depends on the catalytic cysteine of the HECT domain (30). We therefore mutated the active-site cysteine in the HECT domain of a FLAG-tagged version of Nedd4-2s to serine. While N-terminally tagged WT Nedd4-2s remained active in the ΔPTAPP rescue assay, the $C_{801}S$ mutant showed no activity despite WT expression levels (Fig. 1C). Thus, the catalytic site of Nedd4-2s is essential for its ability to rescue HIV-1 budding.

Nedd4-2s overexpression can rescue HIV-1 release in the absence of all known L domains. In addition to the PTAPP-type L domain, HIV-1 p6 harbors an auxiliary L domain of the $LYPx_nL$ type. The $LYPx_nL$ motif interacts with the V domain of ALIX and is required for the ability of ALIX to rescue

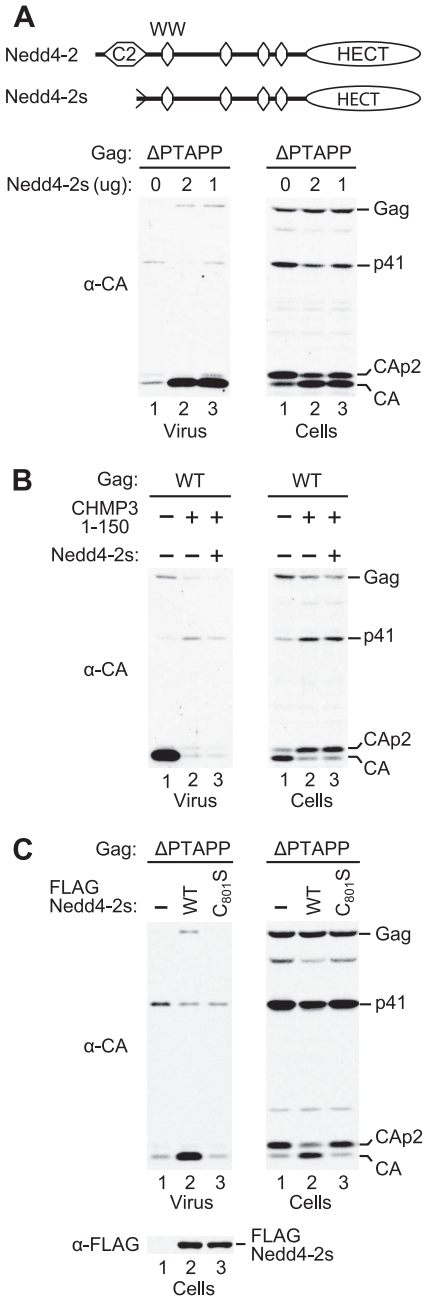

FIG. 1. "Rescue" of a Tsg101-binding-site mutant by Nedd4-2s. (A) The overexpression of Nedd4-2s profoundly increases HIV-1 release and corrects Gag processing in the absence of the PTAPP L domain. 293T cells were transfected with $HXBH10_{\Delta PTAPP}$ (1 μg) and either empty pBJ5 or a version expressing Nedd4-2s. Virion production and Gag expression levels were compared by Western blotting with anti-CA (α-CA) antibody. (B) Exogenous Nedd4-2s does not overcome the inhibition of WT HIV-1 budding by dominant-negative CHMP3. (C) The catalytic site (Cys-801) in the HECT domain of Nedd4-2s is required to enhance budding and Gag processing by $HXBH10_{\Delta PTAPP}$. α-FLAG, anti-FLAG.

Tsg101-binding-site mutants (12, 47). To determine whether the rescue by Nedd4-2s also required the presence of specific p6 sequences, we examined the effect of Nedd4-2s on a proviral mutant (here called Δp6) that encodes a Gag precursor which precisely lacks p6 but can express the overlapping *pol* frame. As shown in Fig. 2A, Nedd4-2s overexpression dramatically increased the release of both CA and CAp2 by the Δp6 mutant, which released essentially no mature CA and only a trace amount of CAp2 in the absence of exogenous Nedd4-2s. Quantitation of the CA and CAp2 bands by densitometry indicated that the effect of Nedd4-2s on the Δp6 mutant was about 75% of that on the ΔPTAPP mutant. Thus, the effect of Nedd4-2s did not critically depend on the ALIX binding site or on any other determinant in p6.

We recently observed that ALIX also binds to the NC domain of HIV-1 Gag (36). We therefore examined the effect of Nedd4-2s on a Gag construct termed $Z_{IL}p1p6_{\Delta PTAPP}$, in which the NC domain is replaced by a variant GCN4 leucine zipper sequence to promote Gag assembly (Fig. 2B). In contrast to the WT GCN4 zipper, the mutant version does not rescue VLP production in the absence of an L domain (1). The overexpression of Nedd4-2s clearly improved VLP production by $Z_{IL}p1p6_{\Delta PTAPP}$, demonstrating that the effect of Nedd4-2s was independent of NC (Fig. 2B).

We also examined the effect of Nedd4-2s on a p6-deleted variant of the Δ8-87/Δ126-277 minimal Gag construct (43). Although the Δ8-87/Δ126-277 mutant lacks both the globular domain of matrix (MA) and the N-terminal domain (NTD) of CA (Fig. 2B), it efficiently forms VLP if p6 is left intact (5). The p6-deleted variant of the Δ8-87/Δ126-277 mutant released only small amounts of VLP, as expected, but VLP production was profoundly increased upon Nedd4-2s overexpression (Fig. 2B). Together, these results indicate that MA, the NTD of CA, NC, and p6 are all dispensable for the ability of Nedd4-2s to stimulate VLP production.

Rescue of HIV-1 L-domain mutant infectivity. ALIX overexpression can dramatically increase the yield of infectious virus released by 293T cells transfected with HIV-1 ΔPTAP (12). We therefore compared the amount of infectious virus produced by 293T cells cotransfected with ΔPTAPP or Δp6 HIV-1 along with either empty vector or the Nedd4-2s expression vector. Remarkably, Nedd4-2s overexpression increased the release of infectious ΔPTAPP virus 40- to 50-fold but did not significantly enhance the titer obtained with WT virus (Fig. 3; also data not shown). While the total infectivity produced by cells expressing $HXBH10_{\Delta PTAPP}$ together with exogenous Nedd4-2s remained about 10-fold lower than the infectivity obtained with WT virus, virus production also remained 2- to 3-fold lower (Fig. 3), indicating that the specific infectivity of ΔPTAPP virions was at most 5-fold reduced from that of WT virions.

While the Δp6 mutant by itself yielded no infectivity, a low level of infectivity became detectable upon Nedd4-2s overexpression (Fig. 3). However, given that Nedd4-2s overexpression largely rescued the release defect of the Δp6 mutant (Fig. 3), the specific infectivity of Δp6 virions remained very low.

Nedd4-2s overexpression does not correct the release defect of an unrelated retrovirus lacking L domains. The ability of Nedd4-2s to stimulate budding by a minimal HIV-1 Gag construct suggested that no specific HIV-1 sequences may be required for this effect. If so, Nedd4-2s overexpression should also stimulate the budding of unrelated retroviruses with defective L domains. We therefore examined the effect of Nedd4-2s on WT and mutant versions of MPMV, a betaretro-

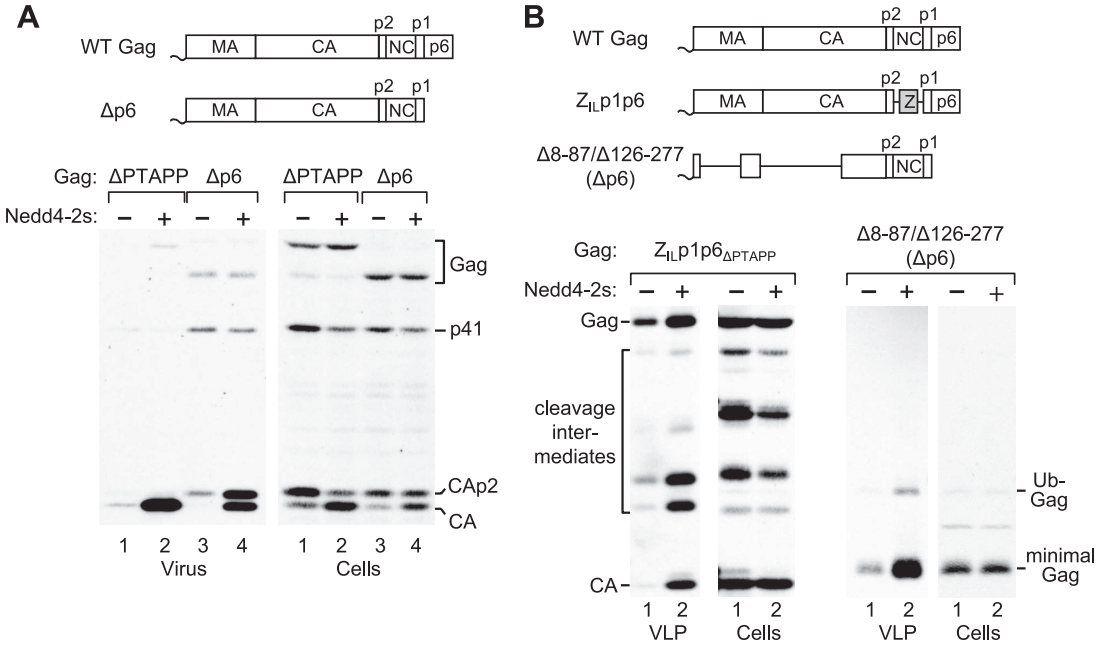

FIG. 2. The effect of Nedd4-2s on HIV-1 budding does not depend on any known L domain. (A) Exogenous Nedd4-2s dramatically enhances the release of mature CA by an HIV-1 mutant lacking all of p6. (B) The effect of Nedd4-2s on VLP release does not depend on NC, MA, or the NTD of CA. $Z_{IL}p1p6_{\Delta PTAPP}$ is protease positive and encodes a Gag polyprotein identical to WT HIV-1 Gag, except that NC is replaced by a leucine zipper and the Tsg101 binding site is deleted. Δ8-87/Δ126-277/Δp6 is protease negative and encodes a minimal assembly-competent Gag molecule that lacks most of MA, all of the NTD, and all of p6.

virus that preassembles immature capsids in the cytoplasm (37). Nevertheless, MPMV budding from the plasma membrane depends on the presence of a PPxY-type L domain in Gag (15, 52). MPMV Gag also contains a PSAP motif that has been shown to interact with Tsg101 (15). The PSAP motif acts as an additional L domain that contributes to the release of MPMV but cannot substitute for the PPPY motif (15).

To examine whether Nedd4-2s can rescue MPMV release defects, we used two previously characterized L-domain mutants (15). The PY(−) mutant has the PPPY motif mutated to PGAA, which is expected to totally abolish WW-domain-mediated interactions with Nedd4 family ubiquitin ligases (30). The 2x(−) mutant additionally has the PSAP motif changed to AGAP and thus lacks a binding site for Tsg101. As previously reported (15), both mutants displayed dramatic defects in viral particle release (Fig. 4). Surprisingly, in contrast to results in previous studies (15, 52), we observed little evidence for an impairment of the intracellular accumulation of mature CA, which may be due to the use of 293T cells in the present study. Also, very little if any unprocessed Gag was detected in the transfected 293T cells, but we cannot exclude that our anti-MPMV CA antiserum recognized the unprocessed Gag precursor only poorly. Interestingly, exogenous Nedd4-2s stimulated the release of the PY(−) mutant but not of the 2x(−) mutant (Fig. 4). Although particle production by the PY(−) mutant remained far below WT levels even upon Nedd4-2s overexpression, these data nevertheless indicate that Nedd4-2s can act through Tsg101. Furthermore, the observation that the 2x(−) mutant was essentially unresponsive to Nedd4-2s implies that the potent effect of the E3 ligase on HIV-1 L-domain mutants is specific.

An intact C2 domain interferes with rescue of HIV-1 ΔPTAPP. A recent study reveals that certain Nedd4 family members, including Smurf2 and Nedd4-1, are autoinhibited through interactions between their C2 and HECT domains (50). Interestingly, full-length Nedd4-2 had a much less robust effect on virus production by $HXBH10_{\Delta PTAPP}$ than Nedd4-2s, even though expression levels were comparable (Fig. 5A, lanes 2 and 3). Furthermore, full-length Nedd4-2 did not at all alleviate the Gag cleavage defect of the ΔPTAPP mutant, whereas Nedd4-2s largely corrected this defect (Fig. 5A). To explore the possibility that Nedd4-2 is autoinhibited, we introduced two mutations into the C2 domain ($IF_{37,38}AA$ and $TL_{69,70}AA$) that target conserved residues implicated in intramolecular interactions between the C2 and HECT domains of Smurf2. Equivalent mutations ($FF_{29,30}AA$ and $TL_{56,57}AA$) have been shown to relieve the autoinhibition of Smurf2 (50). While the $IF_{37,38}AA$ mutant was poorly expressed, the $TL_{69,70}AA$ mutant indeed was somewhat more active than WT Nedd4-2 in rescuing $HXBH10_{\Delta PTAPP}$ (Fig. 5A, lanes 4 and 5). Taken together, these results are consistent with the notion that the C2 domain of Nedd4-2 confers autoinhibition.

Since the presence or absence of an intact C2 domain markedly influenced the activity of Nedd4-2 in the ΔPTAPP rescue assay, we also tested the activities of other Nedd4 family members after deleting the exact portion of the C2 domain that is naturally absent from Nedd4-2s. While WWP1s remained completely inactive in the ΔPTAPP rescue assay (Fig. 5B, lane 2), Nedd4-1s moderately increased particle release but failed to correct Gag processing (lane 3). We thus conclude that the robust activity of Nedd4-2s is a specific property of this particular ubiquitin ligase.

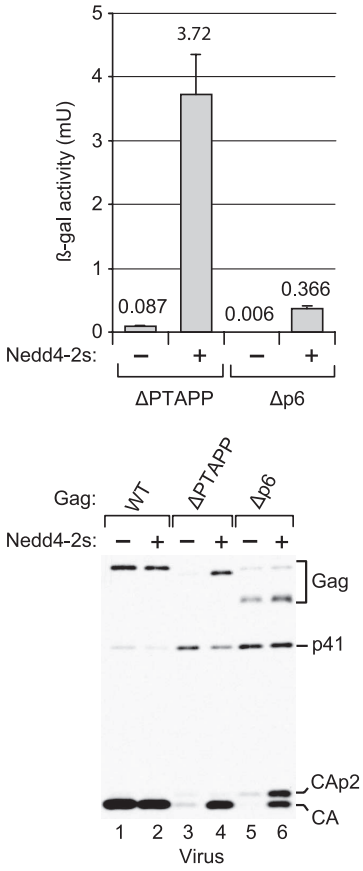

FIG. 3. Nedd4-2s overexpression enhances the release of infectious virus by HIV-1 p6 mutants. Infectious virus produced by 293T cells transfected with ΔPTAPP or Δp6 HIV-1 and empty vector or a vector expressing Nedd4-2s was quantified on TZM-bl indicator cells by measuring the induction of β-Gal by HIV-1 Tat. Values represent the average from three parallel infections, and error bars correspond to one standard deviation. WT HIV-1 expressed in the absence or presence of exogenous Nedd4-2s yielded 37.7 and 44.3 mU of β-Gal activity, respectively. Virus particle production by the transfected 293T cells was analyzed in parallel by Western blotting.

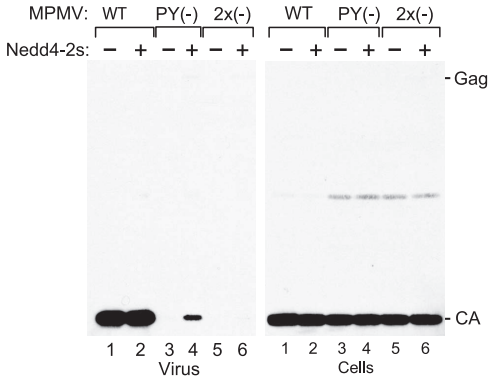

FIG. 4. Ectopic Nedd4-2s does not rescue particle production by MPMV lacking both PPxY and PSAP L domains. 293T cells were cotransfected with WT or mutant pMPMV and empty vector or the Nedd4-2s expression vector. The PY(−) mutant lacks the PPxY motif but retains the PSAP motif, whereas the 2x(−) mutant lacks both motifs. Virion- and cell-associated Gag was detected by Western blotting with goat anti-p27 serum. The position of CA (p27) and the expected position of uncleaved Gag are indicated.

Nedd4-2s rescues HIV-1 ΔPTAPP in the absence of intact WW domains. Nedd4-2 contains four WW domains, which are thought to be responsible for substrate recognition (22). To determine the role of the WW domains in the rescue of HIV-1 ΔPTAPP, each WW domain of Nedd4-2s was individually disrupted by changing a conserved WxxP motif to FxxA. This strategy was based on the observation that these single-amino-acid substitutions are individually sufficient to block ligand binding by the WW domain of Yes-associated protein (7). As shown in Fig. 6A and D, all four WW domains of Nedd4-2s could be individually disrupted without affecting the ability to rescue $HXBH10_{\Delta PTAPP}$. To address the possibility of functional redundancy, we also examined the effects of simultaneously disrupting adjacent WW domains. Again, when the expression levels of the mutants were taken into account, none of the combined mutations tested appeared to affect the activity of Nedd4-2s in the ΔPTAPP assay (Fig. 6B). Therefore, the WxxP-to-FxxA change was introduced into all four WW domains simultaneously (WW1,2,3,4m). This led to a slight reduction in the ability to rescue $HXBH10_{\Delta PTAPP}$, particularly its Gag cleavage defect (Fig. 6C). However, the WW1,2,3,4m quadruple mutant was also less well expressed than WT Nedd4-2s. Compared to the $C_{801}S$ active-site mutant (Fig. 6C, lane 1), two independently generated clones of the WW1,2,3,4m quadruple mutant clearly remained active (lanes 3 and 4). These observations indicate that WW-domain-mediated protein-protein interactions do not play a critical role in the rescue of HIV-1 budding by Nedd4-2s.

Residual C2-domain sequences in Nedd4-2s are essential for the rescue of HIV-1 ΔPTAPP. Since all of the WW domains of Nedd4-2s could be altered without eliminating its activity on HIV-1 budding, we examined the effects of N-terminal truncations that removed either one (Δ1-103), two (Δ1-275), or all four (Δ1-431) of the WW domains (Fig. 6D). We found that the three truncation mutants were all inactive in the ΔPTAPP rescue assay (Fig. 6D, lanes 4 to 6). Similarly, a less-extensive truncation that removed only residues N-terminal to the first WW domain (Δ1-61) eliminated all activity in this assay (Fig. 6D, lane 7). Thus, the four WW domains together with the HECT domain were not sufficient to induce HIV-1 release. This finding contrasts with the observation that fragments of WWP1 or WWP2 that harbored only the WW and HECT domains retained the ability to induce the release of a murine leukemia virus with an attenuated PPxY-type L domain (30).

Although Nedd4-2s lacks an intact C2 domain, it retains the region which forms β-strands 7 and 8 of the C2 domain at its very N terminus (50). Interestingly, an N-terminal truncation mutant that lacked only these residues (Δ1-31) showed no ability to induce the budding of $HXBH10_{\Delta PTAPP}$ (Fig. 6D, lane 8), indicating that the residual C2 domain sequences in Nedd4-2s are essential for this activity.

Residual C2 domain sequences are required for the association of Nedd4-2s with VLP. In addition to its function as a phospholipid binding domain, the C2 domain of Nedd4-1 can mediate protein-protein interactions (38). Since our data indicated that the effect of Nedd4-2s on budding is specific for HIV-1, we explored the possibility that the residual C2 domain of Nedd4-2s allows the E3 ligase to directly or indirectly asso-

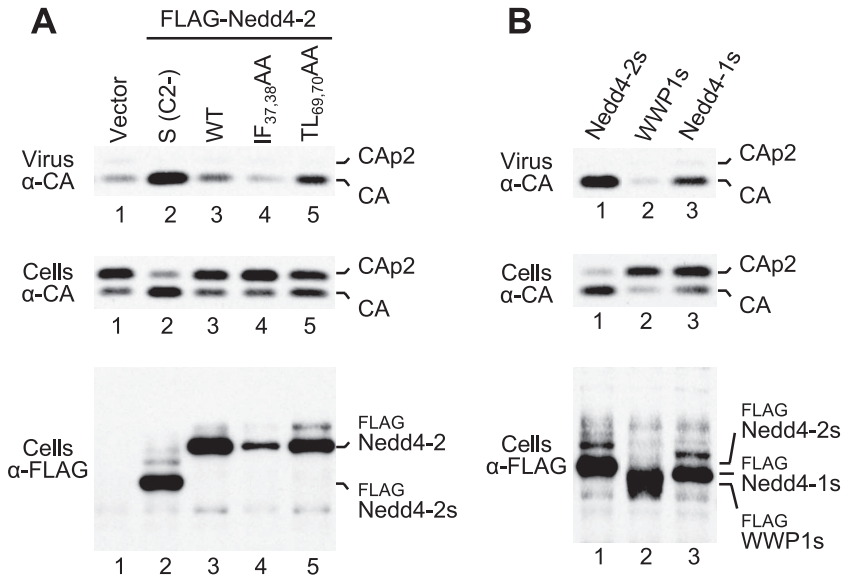

FIG. 5. Role of the C2 domain in the rescue of HIV-1 budding. (A) Nedd4-2 with an intact C2 domain is poorly active in the ΔPTAPP rescue assay, and activity is moderately enhanced by a mutation designed to relieve an autoinhibitory interaction between the C2 and HECT domains. 293 T cells were cotransfected with $HXBH10_{\Delta PTAPP}$ and empty vector (lane 1) or vectors expressing WT Nedd4-2s (lane 2), WT Nedd4-2 (lane 3), $IF_{37,38}AA$ Nedd4-2 (lane 4), or $TL_{69,70}AA$ Nedd4-2 (lane 5). α-CA, anti-CA; α-FLAG, anti-FLAG. (B) Nedd4-2s is more active in the ΔPTAPP rescue assay than equivalent versions of WWP1 or Nedd4-1. 293 T cells were cotransfected with $HXBH10_{\Delta PTAPP}$ and Nedd4-2s (lane 1), WWP1s (lane 2), or Nedd4-1s (lane 3).

ciate with HIV-1 Gag. To this end, we used the level of Gag ubiquitination mediated by the ubiquitin ligase as a surrogate marker to assess the extent of contact between Gag and the E3 ligase. Since neither NC nor p6 is required for the stimulation of VLP release by Nedd4-2s, we examined the effect of Nedd4-2s on the ubiquitination of a Gag construct called Z_{WT}, in which NC and p6 are replaced by a heterologous dimerization domain (1). Z_{WT} Gag does not require an L domain (1), and VLP production was thus not expected to be significantly affected by Nedd4-2s, allowing us to compare the effects of active and inactive versions of Nedd4-2s on VLP-associated Gag.

As shown in Fig. 7A, the coexpression of FLAG-Nedd4-2s led to the appearance of a ladder of Gag species in Z_{WT} VLP that migrated slower than the Z_{WT} Gag precursor (lane 2). As expected for Nedd4-2s-induced Gag-ubiquitin conjugates, the additional Gag species were not obtained with the $C_{801}S$ mutant (Fig. 7A, lane 3). Remarkably, the generation of the putative Gag-ubiquitin conjugates was also markedly reduced by the Δ1-61 and Δ1-31 truncations, even though the truncation mutants were well expressed and appeared to remain capable of autoubiquitination (Fig. 7A, lanes 4 and 5; also see Fig. 6D). On the other hand, the WW1,2,3,4m mutant, which remained active in the ΔPTAPP rescue assay, had an effect on the appearance of Gag-ubiquitin conjugates similar to that of WT Nedd4-2s (Fig. 7B).

To confirm that the Δ1-31 truncation interfered with the ubiquitination of Z_{WT} Gag, the Z_{WT} construct was cotransfected together with vectors expressing WT or mutant FLAG-Nedd4-2s and HA-ubiquitin. Again, WT but not Δ1-31 FLAG-Nedd4-2s induced the appearance of modified Gag species in Z_{WT} VLP that were recognized by the anti-CA antibody (Fig. 8). These extra Gag species comigrated with ubiquitin conjugates detected with the anti-HA antibody, verifying that the Nedd4-2s-induced bands represented Gag-ubiquitin conjugates (Fig. 8). Importantly, the levels of Gag-ubiquitin conjugates obtained with the Δ1-31 mutant were only slightly higher than those obtained with the $C_{801}S$ active-site mutant or the empty vector, confirming that the Δ1-31 truncation largely prevented the ubiquitination of Z_{WT} Gag by Nedd4-2s (Fig. 8).

To obtain more-direct evidence that the removal of residual C2-domain sequences prevented the association of Nedd4-2s with Z_{WT} Gag, we examined the incorporation of FLAG-Nedd4-2s into Z_{WT} VLP. Interestingly, WT FLAG-Nedd4-2s was readily detectable in VLP, whereas the Δ1-61 and Δ1-31 truncation mutants were not (Fig. 7A and 8). This difference was not attributable to differences in expression, since both truncation mutants were expressed at least as well as WT FLAG-Nedd4-2s (Fig. 7A and 8). Also, the WW1,2,3,4m mutation led to higher levels of FLAG-Nedd4-2s in Z_{WT} VLP, in spite of its negative effect on the expression levels of FLAG-Nedd4-2s (Fig. 7B). Thus, the incorporation of FLAG-Nedd4-2s required specific determinants in the E3 ligase, providing evidence for a specific association of Nedd4-2s with Z_{WT} Gag that depends on residual C2-domain sequences but not on intact WW domains.

The active-site cysteine of the HECT domain is critical for association of Nedd4-2s with VLP. Recently a novel mode of interaction between Nedd4-1 and a substrate has been described that does not rely on WW-domain-mediated binding (51). Rather, target recognition is based on an interaction between ubiquitin attached to the E3 ligase and a ubiquitin-binding domain (UBD) of the substrate (51). HIV-1 virions contain substantial amounts of free monoubiquitin, which is unlikely to be derived from Gag-ubiquitin conjugates (34). Thus, it seemed possible that the association of Nedd4-2s with

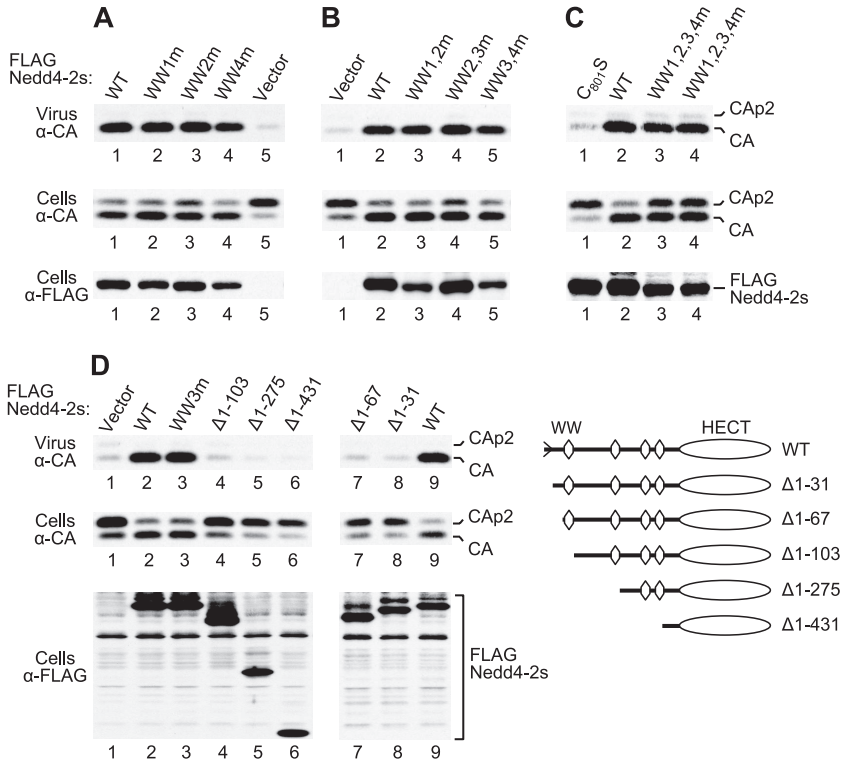

FIG. 6. The rescue of HIV-1 budding by Nedd4-2s does not require any intact WW domain but depends on residual C2-domain sequences. WW domains can be disrupted individually (A and D) or in pairwise combinations (B) without affecting the activity of Nedd4-2s in the ΔPTAPP rescue assay. (C) The ability of Nedd4-2s to rescue $HXBH10_{\Delta PTAPP}$ is only moderately attenuated if all four WW domains are mutated simultaneously. Two independently generated clones of the quadruple mutant (WW1,2,3,4m) were tested to verify reproducibility. (D) The HECT domain of Nedd4-2s alone (lane 6) or various WW-HECT domain fragments (lanes 4, 5, and 7) are not sufficient for activity in the ΔPTAPP rescue assay, and an N-terminal truncation mutant of Nedd4-2s that lacks only the residual C2 domain is also inactive (lane 8).

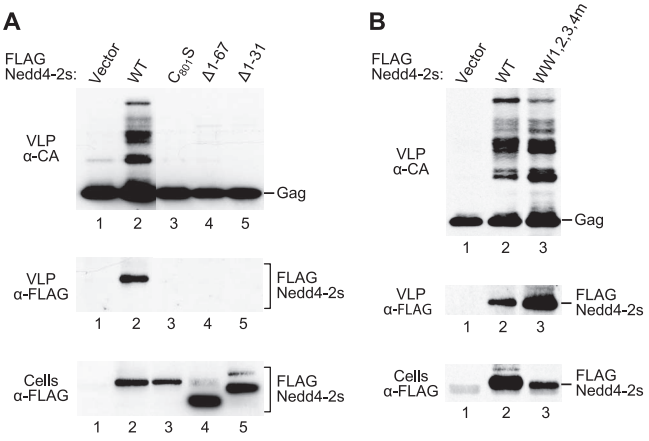

FIG. 7. The residual C2 domain and the HECT domain active site but no intact WW domains are required for the association of Nedd4-2s with VLP. (A) N-terminal truncations that remove residual C2-domain sequences or an active-site mutation impair the modification of an HIV-1 Gag construct by Nedd4-2s and the association of Nedd4-2s with VLP. 293T cells were cotransfected with the L-domain-independent Z_{WT} Gag construct and WT FLAG-Nedd4-2s or the indicated mutants. VLP were analyzed by Western blotting with anti-CA (α-CA) to detect unmodified and modified versions of Z_{WT} Gag and with anti-FLAG (α-FLAG) to detect VLP-associated Nedd4-2s. The cell lysates were also examined with anti-FLAG. (B) Nedd4-2s retains the ability to modify Z_{WT} Gag and to associate with Z_{WT} VLP if all four WW domains are disrupted.

VLP depended on the attachment of ubiquitin to the E3 ligase. In this model, the active-site cysteine of the HECT domain would be expected to be critical for the association, because it is required both for the formation of a thioester bond with ubiquitin and for autoubiquitination. As shown in Fig. 7A and 8, which depict independent experiments, the $C_{801}S$ active-site mutation inhibited the incorporation of FLAG-Nedd4-2s into Z_{WT} VLP, as predicted by the model.

DISCUSSION

Certain Nedd4 family ubiquitin ligases have previously been implicated in the function of PPxY-type L domains (4). However, HIV-1 release was not expected to be stimulated by any of these ligases, since HIV-1 Gag lacks a PPxY motif. In keeping with this expectation, we find that the overexpression of several Nedd4 family ubiquitin ligases with an intact C2 domain has little or no effect on the processing and release of HIV-1 L-domain mutants. In contrast, as shown here and in the accompanying paper by Sundquist and colleagues (8a), a native isoform of Nedd4-2 that lacks most of the C2 domain largely corrected HIV-1 release defects upon overexpression. The ability of Nedd4-2s to rescue budding by Tsg101-binding-site mutants was comparable to that of ALIX (12, 47), although Nedd4-2s was considerably more effective than ALIX in correcting Gag processing defects. Impaired Gag processing is likely to contribute to the several-hundred-fold loss in infec-

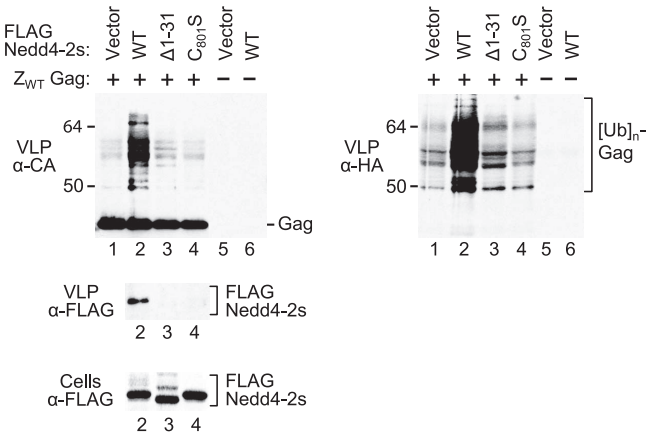

FIG. 8. The removal of residual C2-domain sequences interferes with the ubiquitination of an HIV-1 Gag construct by Nedd4-2s. To detect Gag-ubiquitin conjugates, the Z_{WT} Gag construct was cotransfected with an expression vector for HA-tagged ubiquitin (Ub) and empty pBJ5 or the indicated Nedd4-2s expression vectors (lanes 1 to 4). As a negative control, an HIV-1 proviral construct unable to express Gag was cotransfected together with the HA-Ub expression vector and either empty pBJ5 or a version expressing WT Nedd4-2s (lanes 5 and 6, respectively). Two aliquots of each VLP sample were run simultaneously on the same gel and analyzed by Western blotting with either anti-CA (α-CA) or anti-HA (α-HA) antibodies. The migration positions of molecular mass markers (in kilodaltons) are indicated on the left. The VLP and cell lysates were also analyzed with anti-FLAG antibody to detect Nedd4-2s.

tivity caused by the absence of the PTAP L domain, which exceeds the reduction in virus release by at least an order of magnitude.

The effect of ALIX on HIV-1 release requires the presence of the $LYPx_nL$ auxiliary L domain in p6 (12, 47). In contrast, Nedd4-2s proved capable of stimulating HIV-1 budding even in the absence of p6 and thus of all known HIV-1 L domains. Another region of HIV-1 Gag that may function in virus release is the NC domain, because we have recently observed that HIV-1 NC engages the Bro1 domain of ALIX (36). However, Nedd4-2s remained capable of rescuing a PTAPP mutant after the NC domain was replaced by an unrelated oligomerization domain. The stimulation of HIV-1 budding by Nedd4-2s also did not depend on the MA domain and NTD of Gag, but we cannot exclude a specific requirement for the C-terminal dimerization domain of CA, which is essential for assembly (5, 10). Indeed, a requirement for a specific determinant in HIV-1 is implied by the finding that an MPMV mutant lacking both the PPxY and PSAP L domains was almost completely unresponsive to Nedd4-2s. Interestingly, Nedd4-2s could partially suppress the equally severe budding defect of a mutant that lacked the PPxY L domain but retained the PSAP motif, indicating that Nedd4-2s can potentiate the ability of Tsg101 to function in viral budding. These observations also suggest that Nedd4-2s may act only on Gag proteins that retain at least one auxiliary L domain, which would imply that HIV-1 harbors yet another region involved in virus release that remains to be identified.

The ability of Nedd4-2s to correct defects in HIV-1 budding and Gag processing considerably exceeded that of an isoform with an intact C2 domain. Previous studies have shown that the C2 domain of Nedd4-1 interferes with its ability to downregulate the epithelial Na^+ channel (24, 40), which suggested that the C2 domain negatively regulates the activity of the ubiquitin ligase. Indeed, it was recently reported that interactions between their C2 and HECT domains autoinhibit C2-WW-HECT-domain E3 ligases, such as Smurf2 and Nedd4-1 (50). However, our data indicate that autoinhibition does not fully explain the relatively low activity of the C2-domain-containing Nedd4-2 isoform in the ΔPTAPP rescue assay.

The Nedd4-2s isoform retains a small portion of the C2 domain at its N terminus, which corresponds to β-strands 7 and 8 of the intact domain. This N-terminal region was essential for the rescue of HIV-1 budding by Nedd4-2s, indicating that the C2 domain remnant constitutes a functional domain of the E3 ligase. The C2 domain fragment was also required for the ubiquitination of an HIV-1 Gag construct by Nedd4-2s and for the incorporation of the E3 ligase into VLP. These observations provide evidence for an association with HIV-1 Gag that depends on the residual C2 domain sequences present in the Nedd4-2s isoform. This association does not involve the NC and p6 domains of Gag, because viral particles produced by authentic HIV-1 Gag did not incorporate more Nedd4-2s than VLP formed by a Gag construct that retained only the MA and CAp2 domains (data not shown). Together, our results suggest that the C2 domain remnant was necessary for activity because it was required to bring Nedd4-2s into close contact with Gag.

The ability of Nedd4-2s to suppress HIV-1 L-domain defects depends on the active-site cysteine in the HECT domain, indicating that the ubiquitination of a substrate protein by the E3 ligase is required. Known substrates for Nedd4 family ubiquitin ligases often contain PPxY motifs, which are recognized by the multiple WW domains of the E3 ligases (22). Alternatively, C2-WW-HECT proteins can employ PPxY-containing adaptor molecules to target substrates that lack interaction sites for their WW domains (22, 26). It has also been suggested that Nedd4-1 WW domains can bind to phosphorylated serine or threonine residues to recognize physiological substrates (28). However, despite the well-documented role of the WW domains in substrate recognition, we found that Nedd4-2s remains capable of correcting HIV-1 release defects even if all of its four WW domains are disrupted simultaneously.

Remarkably, the association of Nedd4-2s with VLP depended on the catalytic site in its HECT domain. Since the active-site mutant differed from WT Nedd4-2s merely by the substitution of an oxygen for a sulfur atom, its different behavior likely reflects a role for ubiquitin in the incorporation of the E3 ligase. In support of this notion, HIV-1 Gag appears capable of directing the incorporation of ubiquitin, because HIV-1 virions contain free monoubiquitin at about 10% of the amount of Gag (34, 35). Furthermore, although a small amount of Gag becomes monoubiquitinated in the p6 domain, the level of free ubiquitin in virions does not change if the lysines in p6 are mutated (34). The notion of a ubiquitin-mediated interaction with an E3 ligase is not without precedent, since it has been demonstrated that ubiquitin attached to Nedd4-1 allows the E3 ligase to functionally interact with the UBD of its substrate, eps15 (51). UBD-ubiquitin interactions are typically of low affinity and transitory (18), which may explain why the UBD-mediated recruitment of Nedd4-1 resulted only in the monoubiquitination of eps15 (51). Similarly,

in the present study the overexpression of Nedd4-2s caused only a limited ubiquitination of Gag.

It remains uncertain whether the ubiquitination of Gag itself plays any role in virus budding. On the one hand, the substitution of lysine residues in Gag can have cumulative effects on retrovirus budding, which supports the notion that Gag ubiquitination has a function in virus release (16, 41). On the other hand, it has recently been shown that PPxY-type L domains remain functional in the context of a Gag molecule that entirely lacks lysine residues or a free N terminus to which ubiquitin could be attached (55). An attractive alternative possibility is that the stimulation of HIV-1 budding and Gag processing by Nedd4-2s involves the ubiquitination of a component of the VPS pathway. For instance, the class E VPS protein Hrs is regulated by monoubiquitination (19), which appears to be mediated by Nedd4-1 (25). Whatever the nature of the target, our results narrow the number of potential candidates by establishing that WW-domain-mediated interactions do not play a role in the activity of Nedd4-2s.

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