Human Immunodeficiency Virus Type 1 Gag Engages the Bro1 Domain of ALIX/AIP1 through the Nucleocapsid[▽]

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Human immunodeficiency virus type 1 (HIV-1) and other retroviruses harbor short peptide motifs in Gag that promote the release of infectious virions. These motifs, known as late assembly (L) domains, recruit a cellular budding machinery that is required for the formation of multivesicular bodies (MVBs). The primary L domain of HIV-1 maps to a PTAP motif in the p6 region of Gag and engages the MVB pathway by binding to Tsg101. Additionally, HIV-1 p6 harbors an auxiliary L domain that binds to the V domain of ALIX, another component of the MVB pathway. We now show that ALIX also binds to the nucleocapsid (NC) domain of HIV-1 Gag and that ALIX and its isolated Bro1 domain can be specifically packaged into viral particles via NC. The interaction with ALIX depended on the zinc fingers of NC, which mediate the specific packaging of genomic viral RNA, but was not disrupted by nuclease treatment. We also observed that HIV-1 zinc finger mutants were defective for particle production and exhibited a similar defect in Gag processing as a PTAP deletion mutant. The effects of the zinc finger and PTAP mutations were not additive, suggesting a functional relationship between NC and p6. However, in contrast to the PTAP deletion mutant, the double mutants could not be rescued by overexpressing ALIX, further supporting the notion that NC plays a role in virus release.

Retroviral Gag polyproteins harbor short peptide motifs that promote the detachment of assembled virions from the cell surface and from each other (16, 22, 24, 41, 49, 50, 52). These motifs, which are known as late assembly (L) domains, often remain functional even if moved to a different position within Gag and can be exchanged among unrelated viruses (39, 51). In the case of human immunodeficiency virus type 1 (HIV-1), the primary L domain consists of a conserved P(T/S)AP motif near the beginning of the C-terminal p6 domain of the Gag polyprotein (22, 24). The PTAP motif serves as a binding site for the host protein Tsg101, which is required for the release of infectious virions (21, 31, 47).

Tsg101 is a component of the endosomal sorting complex ESCRT-I, which functions in the sorting of ubiquitinated cargo into small vesicles that bud into the lumen of multivesicular bodies (MVBs) (3, 23, 27). This invagination event is topologically related to retrovirus budding and ultimately serves to deliver transmembrane proteins to lysosomal compartments for degradation. The components of this cellular budding pathway were initially identified in *Saccharomyces cerevisiae*, where a protein network that includes at least 18 different vacuolar protein-sorting (Vps) proteins is required for the formation of MVBs (3). The absence of any of these gene products causes the formation of an abnormal endosomal structure called the class E compartment (26). The MVB sorting pathway is conserved throughout eukaryotic evolution, and at least one homolog for each of the yeast class E Vps proteins exists in humans (48). Most of the class E Vps proteins participate in the formation of ESCRT-I or of two other high-molecular-weight complexes called ESCRT-II and ESCRT-III, which function downstream of ESCRT-I (25). MVB sorting also depends on the activity of the AAA-type ATPase VPS4, another class E Vps protein that recycles the ESCRT machinery (4). Dominant-negative versions of various ESCRT-III subunits and of VPS4 potently inhibit the functions of all known types of viral L domains (21, 30, 43, 44, 48, 53), indicating that all act through components of the MVB sorting pathway.

In addition to the PTAP motif, the p6 domain of HIV-1 Gag contains a LYPx$_n$L motif near its C terminus that binds to ALIX/AIP1 (44), a homolog of the yeast class E Vps protein Bro1 that functions in endosomal trafficking (36). Like Tsg101, ALIX promotes viral egress, which requires the separation of the viral envelope from the plasma membrane, and is also involved in membrane fission events that are necessary for the completion of cytokinesis (10). ALIX has a modular organization, with a banana-shaped N-terminal Bro1 domain, a central domain that is shaped like a V and has therefore been termed the V domain, and a C-terminal proline-rich domain (PRD) that is thought to be unstructured (20, 29). ALIX interacts with the ESCRT-III component CHMP4 through its Bro1 domain (36), with LYPx$_n$L-type viral L domains through its V domain (11, 33), and with Tsg101 and other endocytic proteins through its PRD (36).

The few lentiviruses that are known to lack a PTAP motif all harbor a high-affinity binding site for ALIX in the C-terminal domain of Gag (6, 44). One of these is equine infectious anemia virus, which depends on ALIX for efficient virus release (20, 30, 41, 44, 48). However, in the case of HIV-1, the ALIX binding site in p6 has only an auxiliary role in virus release that becomes more evident in a minimal Gag context (20, 44). Nevertheless, exogenous ALIX can potently rescue the profound budding defect of HIV-1 PTAP mutants, provided that the ALIX binding site in p6 remains intact (20, 46).

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We previously reported that the PTAP-type L domain in HIV-1 p6 does not function autonomously but depends on other Gag regions in order to function (45). Our results suggested that the PTAP motif needs to cooperate either with another determinant in p6, which is now known to bind ALIX, or with the nucleocapsid (NC)-p1 region, which immediately precedes the p6 domain in the context of the Gag precursor (45). The NC domain contains two highly conserved CCHC motifs that coordinate zinc and are required for the selective packaging of genomic viral RNA into progeny virions (5). NC also possesses a nonspecific nucleic acid binding activity that depends on its basic residues and is crucial for Gag polymerization and assembly (13). In contrast, disruption of the two CCHC motifs had no significant effect on Gag-Gag interactions (13).

We now show that NC associates with the Bro1 domain of ALIX through its two CCHC motifs. We also show that the ability of ALIX to rescue a Tsg101 binding site mutant depends on intact CCHC motifs in NC. Disrupting both CCHC motifs led to defects in particle production and to an accumulation of the capsid (CA)-p2 and p41 Gag cleavage intermediates that is typically seen with L domain mutants (21, 22). However, the effects of disrupting the CCHC motifs and the PTAP motif in p6 were not additive, which supports the hypothesis that these motifs function in the same pathway during virus production.

MATERIALS AND METHODS

Proviral constructs. HXBH10, the parental proviral plasmid used in this study, is a *vpu*-positive version of the infectious HXB2 proviral clone of HIV-1. HXBH10-*gag*⁻ is unable to express Gag due to premature termination codons in the *gag* gene (19). The protease (PR)-negative variant HXBH10-PR⁻, which was used to express Pr55^{gag}, and the HXBH10-based chimeric Gag constructs Z_{WT} and Z_{WT}-p6 have been described elsewhere (1). Z_{WT} encodes an HIV-1 Gag precursor that has the NC-p1-p6 region precisely replaced by the GCN4 leucine zipper domain (1). Z_{WT}-p6 has the HIV-1 p6 coding sequence directly fused to the 3′ end of the GCN4 sequence (1). Pr55(Y_{36}s) and Z_{WT}-p6(Y_{36}s) are variants of HXBH10-PR⁻ and Z_{WT}-p6, respectively, that harbor the previously described Y_{36}s mutation (22), which introduces a premature termination codon in place of Tyr-36 of p6 without altering the *pol* frame. The codons for NC-p1 residues 56 to 71, 52 to 71, 36 to 71, and 15 to 71 were precisely deleted from HXBH10-Y_{36}s without affecting any other Gag coding sequences. This was achieved by introducing silent mutations into the first two p6 codons to generate a PstI restriction site. All of these deletions remove the frameshift signal required for the expression of PR and thus prevent Gag processing. In contrast, the Δ15–39, Δ15–34/C_{36}S, Δ15–28, Δ30–31, $C_{28,49}$S, and $K_{33,34}$A mutations keep the frameshift signal intact and were therefore introduced into the PR-negative version of HXBH10-Y_{36}s to prevent Gag processing. The Δ15–39, Δ15–34 C_{36}S, and Δ15–28 mutations have two foreign codons specifying Leu-Gln inserted in place of the deleted NC codons. The $C_{28,49}$S and Δ15–39 mutations were also introduced into the PR-positive proviruses HXBH10 and ΔPTAPP, a variant of HXBH10 with an in-frame deletion of codons 7 through 11 of p6 (1).

Expression vectors. The pBJ5-based mammalian expression vectors for ALIX-HA and HA-ALIX have been described previously (44). The F_{676}D mutant of ALIX-HA was made using the QuikChange mutagenesis strategy (Stratagene). The expression vector for HA-ALIX$_{Bro1}$ was obtained by inserting a PCR product encoding ALIX residues 1 to 362 with an N-terminal hemagglutinin (HA) tag between the NotI and EcoRI sites of pBJ5. The coding sequence for APOBEC3G with a C-terminal FLAG tag was amplified from BC024268 (Open Biosystems) and cloned between the HindIII and XhoI sites of pcDNA3.1(+) (Invitrogen). In all mammalian expression vectors, the start codon is preceded by a Kozak consensus sequence for efficient translation initiation.

Analysis of virus-like particles (VLP). 293T cells (3.5×10^6) or HeLa cells (8×10^5) were seeded into T80 flasks and cotransfected 24 h later with HIV-1 proviral DNA and ALIX expression vectors as indicated using a calcium phosphate precipitation technique. The total amount of transfected DNA was kept

constant with carrier DNA (pBluescript). Twenty-four h (293T) or 48 h (HeLa) posttransfection, the cells were lysed in radioimmunoprecipitation assay buffer (140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), and the culture supernatants were clarified by low-speed centrifugation and passaged through 0.45-μm filters. Virions released into the medium were then pelleted through 20% sucrose cushions. Pelletable material and the cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described elsewhere (1), using a rabbit anti-HIV CA serum (Advanced Biotechnologies) or the anti-HIV CA antibody 183-H12-5C (12) to detect Gag proteins. HA-tagged ALIX was detected with the anti-HA antibody HA.11 (Covance).

GST pull-down assay. PCR fragments encoding the full-length NC (residues 1 to 55) and NC-p1 (residues 1 to 71) domains of HIV-1$_{HXB2}$ Gag were inserted in frame between the EcoRI and XhoI sites of pGEX-4T-1 (GE Healthcare). Glutathione *S*-transferase (GST) fusion proteins were expressed in strain BL21 and immobilized on glutathione-Sepharose beads (GE Healthcare). The beads were then incubated for 3 h at 4°C with hypotonic lysates of 293T cells transiently expressing epitope-tagged ALIX or APOBEC3G, followed by extensive washing in phosphate-buffered saline. Where indicated, the beads were incubated for another 30 min at 37°C in the presence or absence of 75 U (0.75 U/μl) benzonase nuclease (EMD Biosciences) in benzonase buffer (1.2 mM $MgCl_2$, 50 mM Tris-HCl [pH 8.0]). Bound proteins were eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE. Epitope-tagged proteins were detected by Western blotting with anti-HA or anti-FLAG M2 antibody, and GST fusion proteins were visualized with colloidal Coomassie brilliant blue G-250.

RESULTS

NC-dependent incorporation of ALIX into HIV-1 virions. We previously examined the incorporation of ALIX into HIV-1 particles using the chimeric Gag constructs Z_{WT} and Z_{WT}-p6 (1, 44), which have NC-p1 replaced by the GCN4 leucine zipper domain (Fig. 1A). Because the GCN4 zipper domain rescues viral particle production by HIV-1 Gag in the absence of the NC-p1-p6 region (1), this approach allowed us to show that p6 mediates the specific incorporation of ALIX (44). Consistent with this finding, we and others have shown that both full-length ALIX and the isolated ALIX V domain bind to an LYPx$_n$L motif near the C terminus of HIV-1 p6 (20, 29, 33, 44, 48). We therefore expected that the incorporation of ALIX into viral particles formed by authentic HIV-1 Gag would be abolished by the Y_{36}s mutation (22), which converts the codon for Y_{36} of p6 into a premature termination codon and prevents the synthesis of the LYPx$_n$L motif. To test this notion, we coexpressed HA-tagged ALIX with HIV-1 proviral constructs expressing either no Gag, the wild-type (WT) Pr55 Gag polyprotein, or Pr55(Y_{36}s) (Fig. 1A and B). As expected, HA-ALIX was readily detected in virions produced by WT HIV-1 Gag, and the incorporation of HA-ALIX was specific, because its appearance in the particulate fraction depended on the expression of Gag (Fig. 1B, lanes 1 and 2). However, we were surprised to find that the Y_{36}s mutation reduced the uptake of HA-ALIX into viral particles only about twofold (Fig. 1B, lane 3), indicating that in an otherwise-WT HIV-1 Gag context the LYPx$_n$L motif contributes to the recruitment of ALIX but is not essential.

To determine whether the uptake of ALIX in the absence of the LYPx$_n$L motif was mediated by the truncated p6 domain of the Y_{36}s mutant, we introduced the Y_{36}s mutation into Z_{WT}-p6 (Fig. 1A). As previously reported (1, 44), the Z_{WT} chimeric Gag molecule efficiently forms VLP that fail to incorporate coexpressed HA-ALIX (Fig. 1C, lane 1). In contrast, HA-ALIX was readily detectable in VLP produced by Z_{WT}-p6 (Fig. 1C, lane 2), again consistent with previous results (44). Inter-
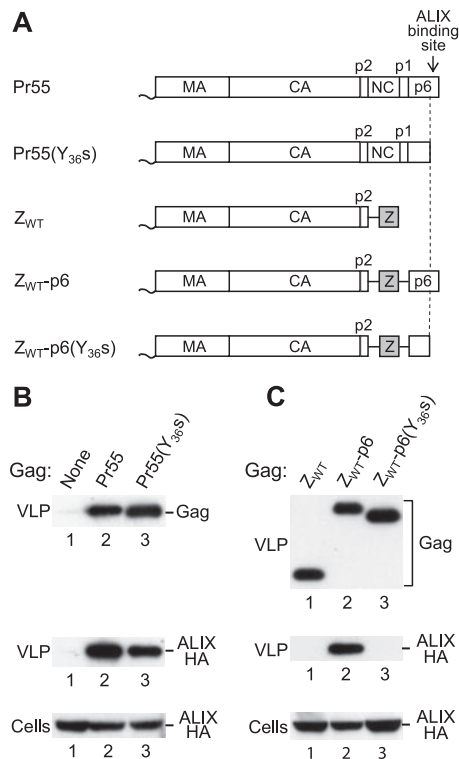
FIG. 1. NC-mediated incorporation of ALIX into VLP. (A) Schematic illustration of the domain organizations of the HIV-1 Gag precursor (Pr55) and of mutant Gag molecules. A wavy line at the N terminus indicates the presence of a myristylation signal, and a gray box indicates the presence of a GCN4 zipper (Z) domain in place of NC-p1. (B) The ALIX binding site in p6 is not essential for the incorporation of ALIX into VLP. 293T cells were transfected with HIV-1 proviral DNA (5 μg) expressing no Gag, WT Gag, or C-terminally truncated Gag, along with 5 μg of a vector expressing ALIX-HA. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and ALIX-HA as indicated. (C) The uptake of ALIX in the absence of the binding site in p6 depends on NC-p1. 293T cells were transfected with HIV-1 proviral DNAs (5 μg) expressing Gag molecules with a leucine zipper in place of NC-p1, along with 5 μg of a vector expressing ALIX-HA.
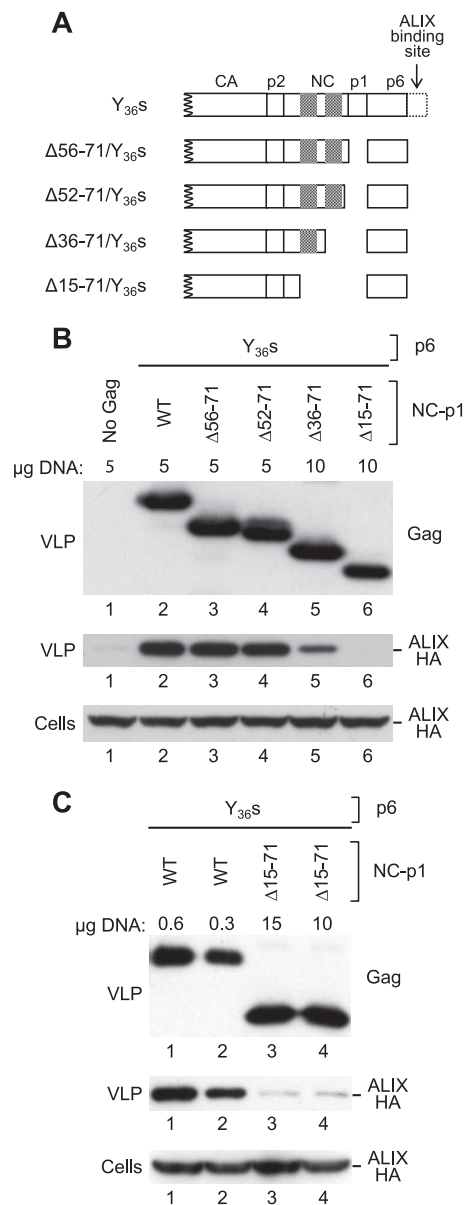

FIG. 2. Effects of deletions in NC-p1 on the uptake of ALIX into VLP. (A) Schematic illustration of the Gag mutants examined. Cross-hatched boxes indicate positions of the zinc fingers in NC. (B) p6-independent incorporation of ALIX depends on NC but not on p1. 293T cells were transfected with the indicated amounts of mutant HIV-1 proviral DNAs together with 3 μg of a vector expressing ALIX-HA. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and ALIX-HA as indicated. (C) Effects of a deletion that removes both zinc fingers of NC together with p1.

estingly, in the context of Z_{WT}-p6, the $Y_{36}s$ mutation abolished the incorporation of HA-ALIX into VLP (Fig. 1C, lane 3). Of note, the $Pr55(Y_{36}s)$ and Z_{WT}-p6($Y_{36}s$) proviral constructs are precisely identical except for the region encoding NC-p1. We thus conclude that the ability of $Pr55(Y_{36}s)$ but not of Z_{WT}-p6($Y_{36}s$) Gag to direct the incorporation of ALIX is dependent on NC-p1.

Role of NC zinc fingers in incorporation of ALIX. The NC domain of HIV-1 Gag harbors two copies of a CCHC-type zinc finger motif that are involved in the specific encapsidation of genomic viral RNA into nascent viral particles. NC also exhibits a nonspecific RNA binding activity that depends on clusters of basic residues and is thought to be critical for virus assembly (8, 13). However, NC deletion mutants are not drastically defective for viral particle production as long as the N-terminal basic NC region proximal to the first CCHC motif is retained (55). Furthermore, it has been shown that particle production by a mutant that lacks nearly all of NC can be rescued by a second site mutation that inactivates PR (38). To maintain

viral particle production, we therefore used a PR-deficient provirus as the parental construct to generate a series of mutants with in-frame deletions in the NC-p1 coding region. Furthermore, all NC-p1 deletions were combined with the $Y_{36}s$ mutation to eliminate the ALIX binding site in the p6 domain of Gag (Fig. 2A).

Since Gag multimerization is concentration dependent (40), we transfected relatively large amounts of these proviral constructs into 293T cells together with a vector expressing ALIX-
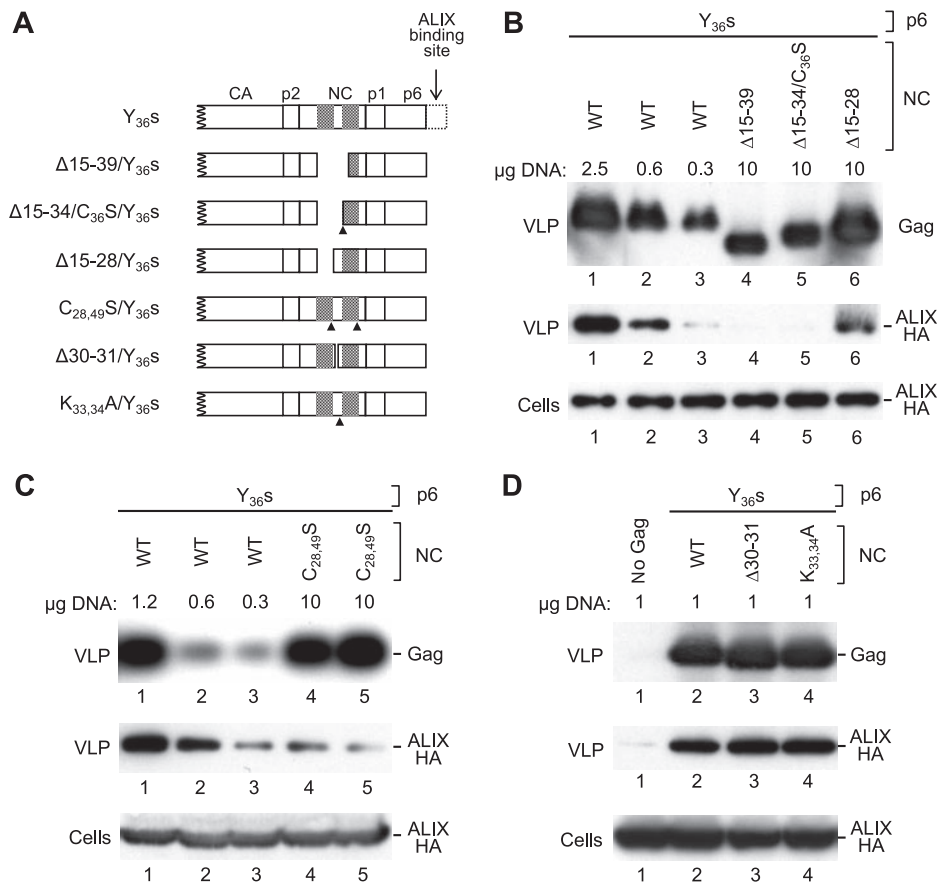
FIG. 3. Role of NC zinc fingers in uptake of ALIX into VLP. (A) Schematic illustration of the Gag mutants examined. Cross-hatched boxes indicate the positions of the zinc fingers in NC, and arrowheads indicate the positions of residues that were substituted. (B) Effects of deletions that specifically target zinc fingers. 293T cells were transfected with the indicated amounts of mutant HIV-1 proviral DNAs, together with 5 μg of a vector expressing ALIX-HA. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and ALIX-HA as indicated. (C) Effect of disrupting both zinc fingers through point mutations. The $C_{28,49}S/Y_{36}s$ mutant was examined in duplicate to verify reproducibility. (D) Effects of mutations in the region between the two zinc fingers.

HA, in an effort to override potential Gag-Gag interaction defects. As a control for Gag-independent release of ALIX in cellular vesicles, the ALIX expression vector was cotransfected with a provirus that lacked the ability to express Gag due to the presence of premature termination codons in the *gag* gene. As shown in Fig. 2B, deleting the region between the second CCHC motif and p6 had no effect on particle production or on the incorporation of ALIX-HA (lanes 3 and 4). In contrast, a deletion which additionally removed the second CCHC motif ($\Delta36-71$) reduced the incorporation of ALIX-HA at least threefold (lane 5). Finally, ALIX-HA was no longer detectable in the particulate fraction if the first CCHC motif was also removed ($\Delta15-71$) (lane 6).

In the experiment shown in Fig. 2B, the $\Delta15-71/Y_{36}s$ mutant at 10 μg produced somewhat fewer VLP than the parental proviral construct at 5 μg. Therefore, the parental construct was titrated down to obtain equivalent levels of particle production. We found that VLP production by the $\Delta15-71/Y_{36}s$ mutant transfected at 10 or 15 μg matched or exceeded that by the parental $Y_{36}s$ provirus only when less than 1 μg of the latter construct was used (Fig. 2C and data not shown). Im-

portantly, this experiment confirmed that $\Delta15-71/Y_{36}s$ VLP incorporate significantly less ALIX than $Y_{36}s$ VLP (Fig. 2C).

Next, we selectively targeted the CCHC motifs in NC to determine their role in the $LYPx_nL$-independent uptake of ALIX. All NC mutants depicted in Fig. 3A harbored the $Y_{36}s$ mutation to prevent the incorporation of ALIX via p6 and a mutation that inactivated PR to suppress potential defects in VLP production in the absence of NC sequences. As shown in Fig. 3B, a deletion that removed the first CCHC motif and a portion of the second ($\Delta15-39$) essentially abolished the ability of the $Y_{36}s$ mutant to incorporate ALIX (compare lanes 2 and 4). A similar effect was observed when a deletion that removed the first CCHC motif and a few flanking residues ($\Delta15-34$) was combined with a point mutation that changed the second CCHC motif to SCHC (lane 5). However, the ability to incorporate ALIX was partially preserved if only the first CCHC motif was deleted and the second was kept intact ($\Delta15-28$) (lane 6). We also note that the latter mutant produced more VLP than those which retained no intact CCHC motif (Fig. 3B). To specifically examine the role of zinc-coordinating residues in the incorporation of ALIX, we changed both CCHC

motifs in NC to CCHS (Fig. 3A). As shown in Fig. 3C, VLP produced by the resulting $C_{28,49}S/Y_{36}s$ mutant reproducibly incorporated less ALIX than those produced by the parental $Y_{36}s$ construct (compare lanes 1 and 2 to lanes 4 and 5). In contrast, mutations that targeted the region between the two CCHC motifs had no effect on the $LYPx_nL$-independent incorporation of ALIX (Fig. 3D). Together, these results indicate that the $LYPx_nL$-independent uptake of ALIX is mediated by the zinc fingers in NC.

ALIX physically associates with HIV-1 NC in vitro. The packaging of ALIX into VLP in the absence of the $LYPx_nL$ motif in p6 suggested that ALIX may also bind to other Gag regions. To examine whether ALIX interacts with the NC-p1 region of HIV-1 Gag, GST-NC or GST-NCp1 was bound to glutathione-Sepharose beads and incubated with extracts from 293T cells expressing FLAG- or HA-tagged ALIX. Epitope-tagged ALIX was readily detected in association with both GST-NC and GST-NCp1, but not with GST alone (Fig. 4A and B). We also observed that the association of ALIX with GST-NCp1 was considerably reduced by the $C_{28,49}S$ mutation (Fig. 4B), consistent with the effect of this mutation on the packaging of ALIX into VLP (Fig. 3C).

We previously showed that the $C_{28,49}S$ double mutation, which disrupts both CCHC motifs in NC, reduces the packaging of genomic viral RNA into HIV-1 virions more than 20-fold (18). We also showed that even the $C_{28}S$ change by itself reduced the binding of viral RNA to HIV-1 Gag 10-fold in a Northwestern blot assay (18). Since the $C_{28,49}S$ mutation also significantly reduced the interaction between NC-p1 and ALIX, we considered the possibility that the in vitro interaction depended on the presence of nucleic acid. To examine this possibility, HA-ALIX was pulled down with GST-NC-p1 bound to glutathione-Sepharose, followed by incubation of the beads with benzonase, a promiscuous nuclease capable of degrading all forms of RNA and DNA. This treatment had no effect on the association of HA-ALIX with GST-NC-p1 (Fig. 4C, lanes 1 to 4). As a control, we examined whether the interaction between NC-p1 and APOPEC3G was affected by benzonase. It was shown previously that the ability of Gag-GST fusion proteins to bind APOBEC3G is inhibited by RNase A (54). Similarly, we observed that benzonase completely disrupted the interaction between GST-NCp1 and APOBEC3G-FLAG under the same experimental conditions in which it had no effect on the interaction with ALIX (Fig. 4C, lanes 5 to 7). Taken together, these results indicate that the interaction between NC or NC-p1 and ALIX is not simply a consequence of unspecific bridging by nucleic acids.

NC engages the Bro1 domain of ALIX. ALIX possesses an N-terminal Bro1 domain and a central V domain that is named for its V-like shape (20, 29). The V domain is required for the binding of ALIX to HIV-1 p6 (44), and recent structural studies revealed that Phe-676 in a conserved hydrophobic pocket of the V domain plays a central role in ALIX-L domain interactions (20, 29). Mutating Phe-676 to Asp abolished the ability of the isolated V domain to inhibit HIV-1 particle release (29) and the ability of full-length ALIX to rescue viral particle production by ΔPTAP HIV-1 (20).

Consistent with these observations, the $F_{676}D$ mutation nearly eliminated the packaging of ALIX into VLP formed by Z_{WT}-p6 (Fig. 5A, compare lanes 5 and 6), which depends on
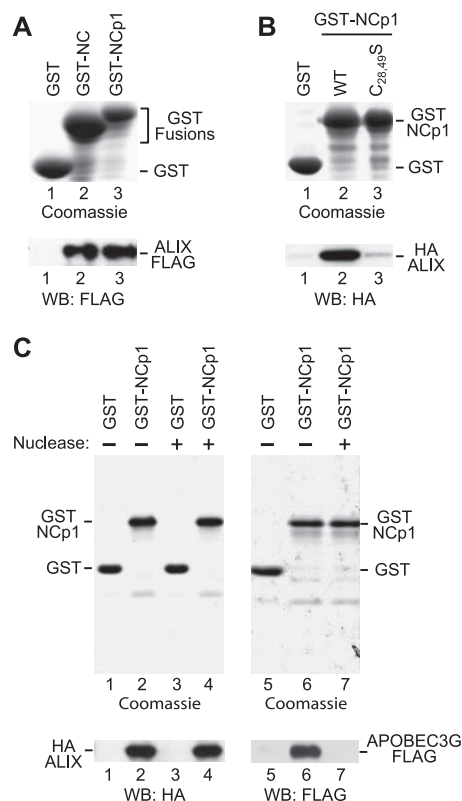

FIG. 4. In vitro interaction between ALIX and NC or NC-p1. (A) ALIX binds to GST-NC and GST-NC-p1. Glutathione-Sepharose beads decorated with bacterially expressed GST or GST fusion proteins were incubated with lysates from 293T cells expressing full-length ALIX with a C-terminal FLAG tag. GST proteins bound to the beads were detected by SDS-PAGE and Coomassie staining, and captured ALIX was detected by Western blotting with anti-FLAG. (B) A GST pull-down assay performed as for panel A, showing that the interaction between ALIX and GST-NC-p1 is impaired by the $C_{28,49}S$ mutation, which disrupts both CCHC motifs in NC. (C) ALIX remains bound to GST-NCp1 in the presence of nuclease, whereas the interaction between GST-NCp1 and APOBEC3G is disrupted. GST pull-down assays were performed as for panel A, followed by incubation of the beads with or without benzonase nuclease. Proteins that remained bound to the glutathione-Sepharose beads were then detected as indicated.

the $LYPx_nL$ motif in p6 (Fig. 1C). In contrast, the same mutation had only a moderate effect on the incorporation of ALIX into particles formed by the WT Pr55 Gag polyprotein (Fig. 5A, lanes 3 and 4). Furthermore, this effect was comparable to that of removing the $LYPx_nL$ motif in p6 on the incorporation of WT ALIX (Fig. 1B). Together, these results suggested that the packaging of ALIX via NC does not depend on the presence of an intact V domain.

We therefore asked whether the isolated Bro1 domain of ALIX can be packaged into VLP. HA-ALIX$_{Bro1}$ was readily detected in VLP produced by the $Y_{36}s$ mutant (Fig. 5B, lane 1), which expresses a Gag polyprotein that lacks the ALIX binding site in p6 but retains the WT NC domain (Fig. 1A). Indeed, HA-ALIX$_{Bro1}$ was incorporated into $Y_{36}s$ mutant VLP at least as well as full-length ALIX (Fig. 5C). In marked contrast, HA-ALIX$_{Bro1}$ was not at all packaged into VLP formed by Z_{WT}-p6 (Fig. 5B, lane 2), which lacks NC but pos-
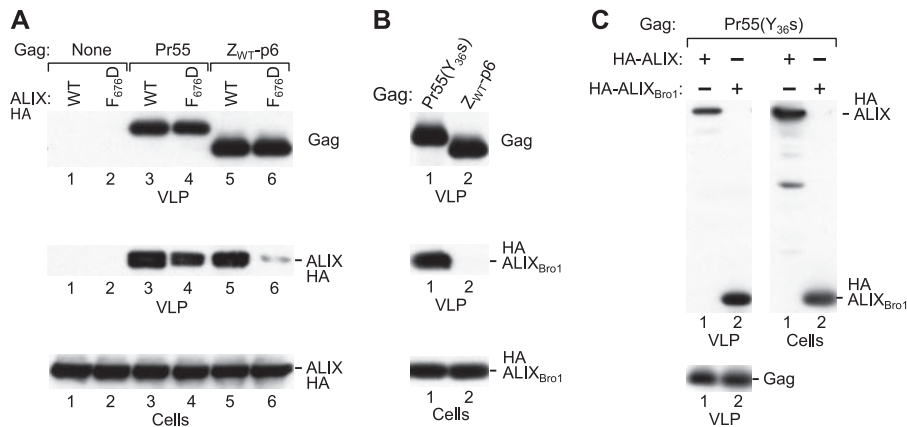
FIG. 5. NC engages the Bro1 domain of ALIX. (A) Effect of disrupting the ligand binding pocket in the V domain on incorporation of ALIX into VLP. 293T cells were transfected with HIV-1 proviral DNAs (7.5 μg) expressing no Gag, WT Gag, or Gag with a leucine zipper in place of NC-p1, along with 5 μg of a vector expressing WT or mutant ALIX-HA. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and ALIX-HA as indicated. (B) NC-dependent incorporation of the isolated Bro1 domain of ALIX. 293T cells were transfected with HIV-1 proviral DNAs (7.5 μg) expressing the indicated Gag molecules, along with 5 μg of a vector expressing HA-ALIX$_{Bro1}$. (C) NC-dependent incorporation of full-length HA-ALIX versus that of HA-ALIX$_{Bro1}$. 293T cells were transfected with HIV-1 proviral DNA (7.5 μg) expressing the Pr55(Y$_{36}$S) Gag precursor, along with 5 μg of vector expressing HA-ALIX or HA-ALIX$_{Bro1}$.

sesses an intact p6 domain (Fig. 1A). This result was not attributable to different levels of VLP production or to differences in the expression of HA-ALIX$_{Bro1}$ (Fig. 5B). We thus conclude that the packaging of HA-ALIX$_{Bro1}$ by the Y$_{36}$S mutant is specific, and we infer that NC interacts with the Bro1 domain of ALIX.

We previously described an HIV-1 mutant which encodes a minimal Gag protein called Δ8–87/Δ126–277 that lacks the globular domain of MA and the N-terminal domain of CA but produces WT levels of VLP (7). L domain function in this minimal Gag context was highly dependent on the presence of an intact ALIX binding site in p6 (44). Moreover, VLP production by ALIX binding site mutants could be rescued by overexpressing ALIX (44). This activity of ALIX depended on the presence of the PTAPP motif in p6, but not on the C-terminal PRD of ALIX (44), which is required for the rescue of ΔPTAPP HIV-1 by ALIX (20, 46). Surprisingly, ALIX$_{ΔPRD}$ markedly increased VLP production even by a variant of Δ8–87/Δ126–277 that harbors the Y$_{36}$S mutation and thus lacks the entire ALIX binding site in p6 (44). We therefore examined whether the V domain of ALIX, which binds to p6 (20, 29), is required for the rescue of VLP production in this system. In both 293T and HeLa cells, HA-ALIX$_{Bro1}$ increased VLP production by the Y$_{36}$S version of the Δ8–87/Δ126–277 minimal Gag construct more than 10-fold without affecting Gag expression levels (Fig. 6A). As expected, HA-ALIX$_{Bro1}$ did not increase the release of ΔPTAPP HIV-1 (Fig. 6B, lanes 1 and 2). Furthermore, HA-ALIX$_{Bro1}$ did not augment virion production by WT HIV-1 (Fig. 6B, lanes 3 and 4). Since the Δ8–87/Δ126–277/Y$_{36}$S minimal Gag construct retains the NC domain, these results raise the possibility that ALIX and the isolated Bro1 domain can act through NC.

NC is required for the function of ALIX in HIV-1 budding. We and Sundquist and coworkers recently reported that the release defects of HIV-1 L domain mutants can be corrected by increasing the cellular expression levels of ALIX (20, 46). For instance, exogenous ALIX completely rescued particle

production by a mutant termed ΔPTAPP, which lacks the Tsg101 binding site in p6 (46). In this ΔPTAPP rescue assay, the activity of ALIX was dependent on the presence of an intact ALIX binding site near the C terminus of p6 (20, 46).

To determine whether NC also plays a role in the rescue by ALIX, we generated a variant of the ΔPTAPP mutant that harbors the C$_{28,49}$S mutation in NC, which interferes with the interaction between NC and ALIX (Fig. 3 and 4). Of note, in a previous study the C$_{28,49}$S mutation had no effect on Gag-Gag interactions (13). Nevertheless, the C$_{28,49}$S mutation by itself reduced viral particle production in 293T cells, although this defect was relatively mild compared to that caused by the ΔPTAPP mutation (Fig. 7A). The C$_{28,49}$S mutation also affected the pattern of cell-associated Gag proteins, which was altered in a manner similar to that seen with the ΔPTAPP mutant. Specifically, both mutations altered the ratio of mature CA versus the CA-p2 processing intermediate and caused an accumulation of the p41 cleavage intermediate, as is typically seen if a late assembly step is defective (21, 22). Interestingly, the C$_{28,49}$S/ΔPTAPP double mutant was no more defective for particle production than the original ΔPTAPP mutant and produced a pattern of cell-associated Gag products similar to that seen with the two parental mutants (Fig. 7A). When an expression vector for ALIX was cotransfected, we observed a profound increase in viral particle production by the ΔPTAPP mutant (Fig. 7B), as previously described (46). In contrast, particle production by the C$_{28,49}$S/ΔPTAPP double mutant was only marginally improved (Fig. 7B).

We also examined the effect of the Δ15–39 deletion in the ΔPTAPP rescue assay, since the Δ15–39 deletion appeared to completely prevent the recruitment of ALIX via NC (Fig. 3B). In the presence of WT PR, the Δ15–39 deletion in NC caused as profound a defect in viral particle production as the ΔPTAPP L domain mutation and also had a comparable effect on the processing of cell-associated Gag (Fig. 8A, lanes 2 and 3). Remarkably, when the two mutations were combined, there again was no additive effect on particle production or on Gag
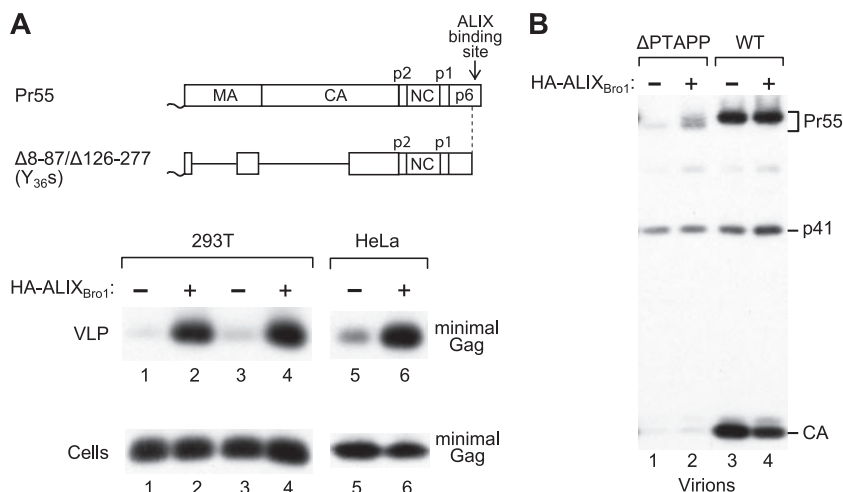
FIG. 6. Effect of HA-ALIX$_{Bro1}$ on particle production. (A) Rescue of VLP production by a minimal Gag construct that lacks the ALIX binding site in p6. 293T cells (lanes 1 to 4) or HeLa cells (lanes 5,6) were transfected with 1 μg (lanes 1,2), 2 μg (lanes 3,4), or 7.5 μg (lanes 5,6) of proviral DNA expressing the Y$_{36}$S version of the Δ8–87/Δ126–277 minimal Gag molecule (44), along with 2 μg (lanes 1 and 2) or 5 μg (lanes 3 to 6) of empty pBJ5 or the HA-ALIX$_{Bro1}$ expression vector. VLP production and Gag expression levels were examined by Western blotting with anti-CA antibody 183-H12-5C. The Δ8–87/Δ126–277 minimal Gag molecule lacks the globular domain of MA and the N-terminal domain of CA, and the Y$_{36}$S truncation removes the ALIX binding site in p6. (B) HA-ALIX$_{Bro1}$ does not increase virion production by ΔPTAPP or WT HIV-1. HeLa cells were transfected with 7.5 μg of ΔPTAPP or WT HXBH10, along with 5 μg of empty pBJ5 or the HA-ALIX$_{Bro1}$ expression vector, and virion production was examined as above.

processing (Fig. 8A, lane 4). Moreover, particle production by the double mutant could not be rescued at all by exogenous ALIX (Fig. 8B). Taken together, these results indicate that the determinants in NC which mediate the interaction with ALIX also play a critical role in the function of ALIX in virus budding.

DISCUSSION

Several studies have shown that ALIX functionally interacts with the p6 domain of HIV-1 Gag (20, 29, 33, 44, 46, 48). We now report that ALIX also interacts with the NC-p1 region, which precedes the p6 domain in the context of the unproc-

essed Gag polyprotein. As a consequence, the specific incorporation of ALIX into HIV-1 virions is only moderately affected by removing its binding site in p6. While we initially identified ALIX based on its p6-dependent incorporation into HIV-1 VLP, this result is explained by the fact that the Gag constructs used in our previous study had NC-p1 replaced by the GCN4 leucine zipper (44). Indeed, the present study confirms that the incorporation of ALIX into VLP becomes completely dependent on the LYPx$_n$L motif in p6 if NC-p1 is replaced by a heterologous dimerization domain.

While the interaction with p6 is mediated by the V domain of ALIX (20, 29, 33), the association with NC was insensitive to
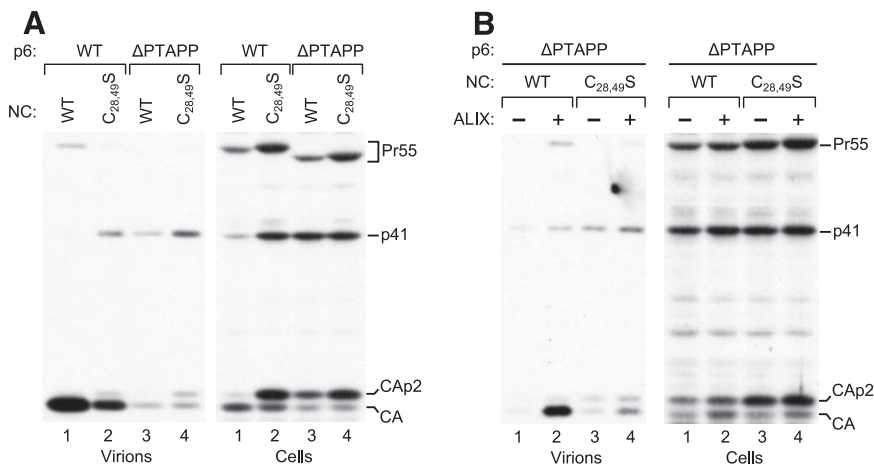

FIG. 7. Effects of disrupting the two NC zinc fingers on particle production and on the ability of ALIX to rescue virus release. (A) Effects of the C$_{28,49}$S mutation on particle production and Gag processing in the presence or absence of the PTAPP L domain. 293T cells were transfected with 1 μg of PR-positive HIV-1 proviral DNAs, and virion production and the pattern of cell-associated Gag products were compared by Western blotting with anti-CA serum. (B) Effect of the C$_{28,49}$S mutation on the ability of ALIX to rescue ΔPTAPP HIV-1. 293T cells were transfected with 1 μg of PR-positive HIV-1 proviral DNAs and either empty pBJ5 or a version expressing HA-ALIX (2 μg).
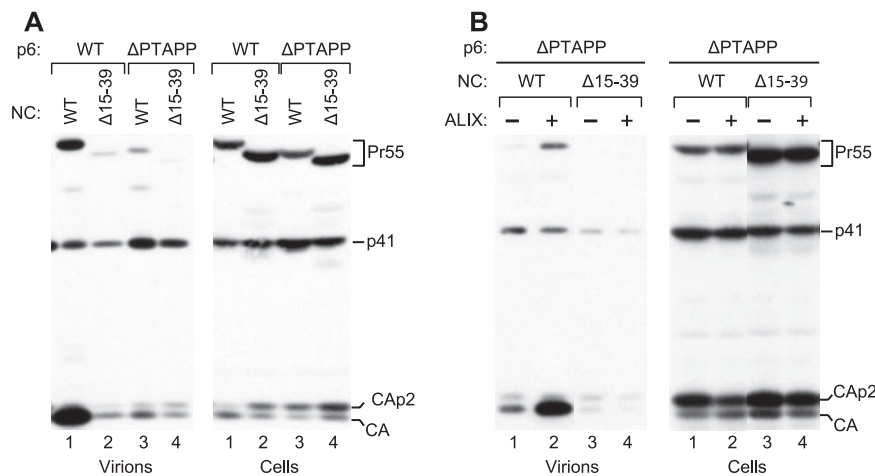
FIG. 8. Effects of removing the NC zinc fingers. (A) The Δ15–39 deletion in NC, which removes all of the first zinc finger and part of the second, and the ΔPTAPP deletion in p6 have comparable effects on particle production and Gag processing, and these effects are not additive. (B) The Δ15–39/ΔPTAPP double mutant is not rescued by ALIX. These experiments were performed as described in the legend for Fig. 7.

a mutation in the ligand binding pocket of the V domain that disrupted binding to p6. Furthermore, the isolated Bro1 domain of ALIX was readily incorporated into viral particles in an NC-dependent manner, indicating that ALIX associates with NC through its N-terminal Bro1 domain. Thus, our results imply that ALIX can interact with HIV-1 Gag through both of its folded domains. The Bro1 domain of ALIX also interacts with ESCRT-III component CHMP4, and mutagenic analyses strongly suggest that this interaction is essential for the function of ALIX in virus budding (20, 46). For the interaction with NC to be functional, ALIX would thus have to be able to accommodate CHMP4 and NC simultaneously. In support of this notion, the overexpression of CHMP4B had no negative effect on the NC-dependent packaging of ALIX into HIV-1 virions (data not shown).

The two CCHC boxes in NC are thought to mediate sequence-specific interactions with the genomic viral RNA by binding to exposed guanosine bases in the packaging signal (2, 15). In the present study, we found that the CCHC boxes also mediate the packaging of ALIX into virions. In particular, substituting zinc-coordinating residues in both CCHC boxes simultaneously nearly abolished the LYPx$_n$L-independent incorporation of ALIX and the in vitro interaction between ALIX and NC-p1. The same modification has been shown to cause a dramatic defect in genomic RNA packaging (18) and to drastically reduce the incorporation of the RNA binding protein Staufen into HIV-1 particles (32). While these parallels point to a relationship between the packaging of ALIX and of the viral genome, there is no evidence that ALIX binds nucleic acid. Moreover, we observed that the in vitro interaction between ALIX and NC-p1 was insensitive to nuclease treatment. This finding indicates that nucleic acid is not required to maintain the interaction and that specific protein-protein contacts are thus likely to be involved.

The interaction of NC with RNA plays an important role in Gag multimerization and in virus assembly, apparently because RNA is needed as a scaffold to promote Gag-Gag interactions (9, 13, 35, 55). However, it is thought that this function of NC involves its nonspecific RNA binding activity, which is primar-

ily mediated by basic residues and does not require intact zinc fingers (8, 13, 14, 17, 37, 42, 55). Nevertheless, we have observed that the C$_{28,49}$S mutation, which disrupts both zinc fingers and interferes with ALIX binding, reduces particle production in HeLa cells about 10-fold (18). This observation contrasts with the finding that the C$_{28,49}$S mutation had no effect on Gag-Gag interactions in an in vitro assay and only moderately affected the nonspecific RNA binding activity of NC (13). In the present study, the C$_{28,49}$S mutation also reduced particle production in 293T cells, albeit to a lesser extent than what we had previously observed in HeLa cells (18). However, a deletion which affected both zinc fingers but left the rest of NC intact reduced particle production severely. Interestingly, these zinc finger mutations had little or no effect on the residual ability of the ΔPTAPP mutant to produce viral particles, which suggests that the mutations in NC and in p6 affected the same step in virus assembly. Consistent with this possibility, the zinc finger mutations caused a similar Gag-processing defect as the ΔPTAPP mutant. These observations point to a role of HIV-1 NC in virus release, in addition to its well-documented function in virus assembly. Of note, a function of NC in virus release is also suggested by the late budding defect of certain NC mutants of the gammaretrovirus Moloney murine leukemia virus (34). Moreover, budding defects strikingly similar to those of L domain mutants have been observed upon deletion of either of the two CCHC motifs in the NC domain of an alpharetrovirus (28).

We previously presented evidence indicating that in order to promote efficient virus release, the conserved PTAPP L domain core of HIV-1 needs to cooperate either with the ALIX binding site in p6 or with NC-p1 (45). Interestingly, the release defect of PTAPP mutants can be fully rescued by overexpressing ALIX, but only if the ALIX binding site in p6 remains intact (20, 46). In the present study, we observed that this effect of ALIX also depended on the zinc fingers in NC, and specifically on determinants in NC that mediate the interaction with ALIX. One interpretation of these results is that the interactions of ALIX with NC and with p6 are both involved in its function in virus budding. However, it also remains possible

that the role of ALIX in virus release strictly depends on the ability of NC to nucleate assembly and that zinc finger mutations affect this ability in a manner that has not been readily apparent in in vitro Gag multimerization assays (13). In either case, the involvement of NC in the activity of ALIX supports the notion that NC has a specific role in virus release.

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