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Divergent Bro1 Domains Share the Capacity To Bind Human Immunodeficiency Virus Type 1 Nucleocapsid and To Enhance Virus-Like Particle Production▽

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To promote the release of infectious virions, human immunodeficiency virus type 1 (HIV-1) exploits the endosomal sorting complex required for transport (ESCRT) pathway by engaging Tsg101 and ALIX through late assembly (L) domains in p6 Gag. An $LYPx_nL$ motif in p6 serves as docking site for the central V domain of ALIX and is required for its ability to stimulate HIV-1 budding. Additionally, the nucleocapsid (NC) domain of Gag binds to the N-terminal Bro1 domain of ALIX, which connects ALIX to the membrane-deforming ESCRT-III complex via its CHMP4 subunits. Since the isolated Bro1 domain of ALIX is sufficient to markedly stimulate virus-like particle (VLP) production in a minimal Gag rescue assay, we examined whether the Bro1 domains of other human proteins possess a similar activity. We now show that the Bro1 domain-only protein Brox and the isolated Bro1 domains of HD-PTP and rhophilin all bind to HIV-1 NC. Furthermore, all shared the capacity to stimulate VLP production by a minimal HIV-1 Gag molecule, and Brox in particular was as potent as the Bro1 domain of ALIX in this assay. Unexpectedly, Brox retained significant activity even if its CHMP4 binding site was disrupted. Thus, the ability to assist in VLP production may be an intrinsic property of the boomerang-shaped Bro1 domain.

Retroviruses engage an endosomal budding machinery via so-called late assembly (L) domains in Gag to promote virus budding at the plasma membrane (4, 17, 33). In the case of human immunodeficiency virus type 1 (HIV-1), the C-terminal p6 domain of Gag harbors a conserved P(T/S)AP motif, which binds to the host protein Tsg101 and functions as the primary L domain (18, 29, 44). Additionally, HIV-1 p6 contains an auxiliary L domain of the $LYPx_nL$ type, which serves as a docking site for ALIX (28, 41, 45). Tsg101 and ALIX are both components of a protein network that is required for the biogenesis of multivesicular bodies (MVB) (22, 38). These compartments are formed through the budding of vesicles from the limiting membrane of endosomes into their lumen, a process that is topologically equivalent to virus budding at the plasma membrane. Recently, it emerged that the protein network essential for MVB formation also functions in cytokinesis, which requires a membrane fission event of similar topology (7, 32).

Most of the components of the protein network that mediates these events are subunits of heteromeric endosomal sorting complexes required for transport (ESCRT) (3, 22, 38). For instance, Tsg101 is a subunit of the heterotetrameric ESCRT-I complex (22, 38). ESCRT-I and the downstream ESCRT-II are stable complexes, whereas ESCRT-III assembles only upon membrane binding (38). ESCRT-III is formed by the structurally related human CHMP proteins, which exist in an autoinhibited monomeric conformation in the cytosol (40, 46). A conformational change from a closed to an open conformation is thus likely required for the activation of CHMP proteins and the assembly of ESCRT-III. Interestingly, the uncontrolled activation of CHMP proteins through the removal of autoinhibitory C-terminal sequences results in the potent inhibition of HIV-1 budding, indicating a central role for ESCRT-III in retroviral release (46).

ALIX consists of a boomerang-shaped N-terminal Bro1 domain, a central ligand binding domain that is shaped like a V, and a C-terminal proline-rich region (16). While ALIX is essential for equine anemia virus budding, its role in HIV-1 budding is less critical than that of Tsg101 (8, 16, 28, 41). However, ALIX can clearly support efficient HIV-1 budding, because its overexpression potently rescues the release defect of Tsg101 binding site mutants (16, 43). This effect of ALIX depends on the interaction between its central V domain and the $LYPx_nL$ motif in HIV-1 p6 (16, 43), confirming that this motif constitutes a functional L domain.

The Bro1 domain of ALIX interacts tightly with ESCRT-III subunit CHMP4B and less avidly with CHMP4A and CHMP4C (25, 28, 41, 45). The ability of ALIX to rescue HIV-1 L domain mutants depends on the interaction between its Bro1 domain and CHMP4, indicating that CHMP4 is of particular importance in viral budding (16, 43). Interestingly, human CHMP4A assembles into membrane-attached filaments if overexpressed in mammalian cells, and these filaments can be induced to form circular arrays that drive the formation of buds and tubules with the same topology as that of a retroviral bud (21). Also, the single yeast ortholog of the mammalian CHMP4 proteins forms homo-oligomeric filaments on endosomes that appear to drive MVB sorting and biogenesis (42).

By binding to membranes with its convex surface, the Bro1

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domain of ALIX could also contribute directly to the generation of negative curvature required for budding away from the cytosol. In support of this notion, we recently observed that the isolated Bro1 domain of ALIX can potently enhance the formation of virus-like particles (VLP) by a minimal HIV-1 Gag construct that retains the primary L domain but lacks certain assembly domains and thus is presumably defective in its ability to deform membranes (37). We also observed that the Bro1 domain of ALIX physically interacts with the nucleocapsid (NC) region of HIV-1 Gag and that mutations in NC that interfere with the interaction induce a phenotype that resembles that of L domain mutants (37).

Despite limited sequence homology between human ALIX and a yeast counterpart, the structures of their Bro1 domains are largely superimposable (16, 26), suggesting that all Bro1 domains have a shape that would be compatible with a membrane-deforming function. We therefore asked whether the ability to stimulate VLP production is unique to the Bro1 domain of ALIX or a property of Bro1 domains in general. We now show that widely divergent Bro1 domains share the ability to associate with HIV-1 Gag in an NC-dependent manner and to enhance VLP production by a minimal Gag molecule. In particular, a human Bro1 domain-only protein termed Brox (23) was as potent as the ALIX Bro1 domain in stimulating VLP production, and even forms of Brox that did not bind to CHMP4 retained significant activity. We thus propose that Bro1 domains are inherently capable of promoting budding events away from the cytosol.

MATERIALS AND METHODS

Proviral constructs. All HIV-1 Gag constructs used in this study were based on HXBH10, a *vpu*-positive version of the infectious HXB2 proviral clone of HIV-1. 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 previously (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) is a variant of HXBH10-PR$^-$ that harbors the previously described Y_{36}s mutation (19), which introduces a premature termination codon in place of Tyr-36 of p6 without altering the *pol* frame. The Δ15–39 and $C_{28,49S}$ versions of the Pr55(Y_{36}s) construct have been described previously (37). The Δ15–39 mutation removes the first zinc finger of NC and a portion of the second, and the $C_{28,49S}$ mutation replaces an invariant Cys in both zinc fingers. The Δ15–49 version of the Pr55(Y_{36}s) construct precisely lacks the two zinc fingers of NC together with the intervening linker region. The Δ15–39 and Δ15–49 mutants have two foreign codons specifying Leu-Gln inserted in place of the deleted NC codons. A version of the Δ8–87/Δ126–277 minimal HIV-1 Gag construct (5) that harbors the Y_{36}s premature termination codon in p6 has also been described (41). HXBH10-*gag*$^-$, which is unable to express Gag due to premature termination codons in the *gag* gene (13), was used as a negative control in all incorporation experiments. The ΔPTAPP version of HXBH10 lacks codons 7 through 11 of p6 (1).

Expression vectors. The pBJ5-based expression vector for HA-$ALIX_{Bro1}$ has been described previously (37). The coding sequence for Brox with or without an N-terminal hemagglutinin (HA) tag was amplified from BC113637 (Open Biosystems) and cloned between the NotI and EcoRI sites of pBJ5. HA-Brox point mutants were made using the QuikChange mutagenesis strategy (Stratagene). To obtain expression vectors for the Bro1 domains of His domain phosphotyrosine phosphatase (HD-PTP) and of rhophilin 1 (RPH1), HD-PTP codons 1 to 364 and RPH1 codons 104 to 501 with an N-terminal HA tag were amplified from BC089042 and BC025767, respectively. The PCR fragments were then cloned between the XhoI and EcoRI sites of pBJ5. The pBJ5-based expression vector for CHMP4B-FLAG was described elsewhere (41). In all mammalian expression vectors, the start codon is preceded by a Kozak consensus sequence for efficient translation initiation.

VLP analysis. 293T cells were seeded into T80 flasks (3.5×10^6 cells) for incorporation experiments, or into T25 flasks (1.2×10^6 cells) to examine effects on particle production. The next day, the cells were cotransfected with HIV-1 proviral DNA and pBJ5-based expression vectors as indicated, using a calcium phosphate precipitation technique. The total amount of transfected DNA was kept constant with carrier DNA (pBluescript). At 24 h 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 or VLP released into the medium were then pelleted through 20% sucrose cushions by ultracentrifugation for 2 h at 27,000 rpm and 4°C in a Beckman SW41 rotor. Pelletable material and the cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described elsewhere (1), using the anti-HIV CA antibody 183-H12-5C (9) to detect Gag proteins. HA-tagged proteins were detected with the anti-HA mouse monoclonal antibody HA.11 (Covance). Western blots were quantitated with the ImageJ software.

GST pulldown assay. Bacterial expression vectors for glutathione *S*-transferase (GST)-NC and GST-NCp1 have been described previously (37). 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 HA-Brox, HA-$RPH1_{Bro1}$, or HA-HD-PTP_{Bro1}. Next, the beads were extensively washed in phosphate-buffered saline, and 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 antibody, and GST fusion proteins were visualized with colloidal Coomassie brilliant blue G-250.

Coimmunoprecipitations. 293T cells (3.5×10^6) were seeded into T80 flasks and cotransfected with expression vectors for HA-tagged wild-type (WT) or mutant Brox and FLAG-tagged CHMP4B (8 μg each). At 24 h posttransfection, the cells were lysed in NP-40 buffer (0.5% NP-40, 20 mM Tris HCl [pH 7.4], 150 mM NaCl, and protease inhibitor cocktail [Complete; Roche Molecular Biochemicals]). The lysates were centrifuged at $16,000 \times g$ and immunoprecipitated for 2.5 h at 4°C with mouse monoclonal anti-HA antibody HA.11. Immunoprecipitates were analyzed by immunoblotting with rabbit polyclonal anti-HA antibody HA.11 (Covance) and with anti-FLAG antibody M2.

RESULTS

Specific incorporation of Brox into HIV-1 particles. We recently reported that HIV-1 NC interacts with the N-terminal Bro1 domain of ALIX (37). To determine whether NC has the capacity to interact with other Bro1 domain-containing proteins, we examined the incorporation of Brox into HIV-1 particles. Human Brox is a 411-amino-acid protein that is composed largely of a Bro1-like domain followed by a C-terminal prenylation site (23). Thus, despite very limited sequence identity, native Brox is expected to structurally resemble the isolated Bro1 domain of ALIX, which is efficiently incorporated into HIV-1 particles if expressed by itself (37). To examine whether Brox is incorporated into HIV-1 particles, we expressed an HA-tagged version together with HIV-1 proviral constructs expressing either no Gag or the WT Pr55 Gag polyprotein. As shown in Fig. 1, HA-Brox was essentially absent from the particulate fraction if no Gag was expressed but was readily detected in viral particles produced by WT HIV-1 Gag (lanes 1 and 2).

The incorporation of ALIX into HIV-1 is mediated by the C-terminal NC-p1-p6 region of Gag (37). To determine whether the same region is required for the incorporation of HA-Brox, we used the chimeric Z_{WT} Gag construct. As illustrated in Fig. 1, Z_{WT} has the NC-p1-p6 region replaced by a heterologous dimerization domain that is sufficient to drive efficient Gag assembly and release (1). HA-Brox was not incorporated into VLP produced by Z_{WT} (Fig. 1, lane 3), even though VLP production was comparable to that obtained in

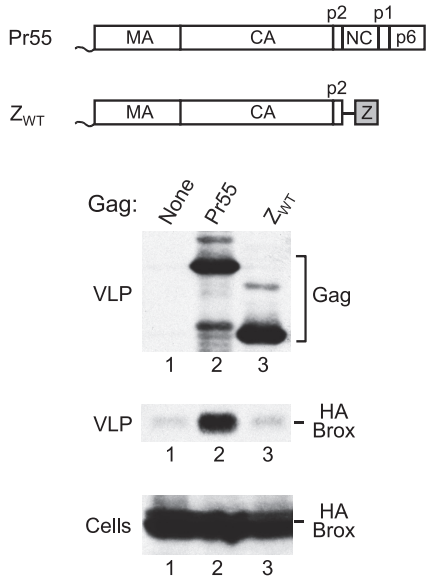

FIG. 1. NC-p1-p6-mediated incorporation of Brox into VLP. 293T cells were transfected with 5 μg of a vector expressing HA-Brox, along with HIV-1 proviral DNA (7.5 μg) expressing no Gag, WT Gag, or a chimeric Gag with a leucine zipper in place of NC-p1-6. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and HA-Brox as indicated.

the presence of intact HIV-1 Gag (compare lanes 2 and 3). Thus, HA-Brox was specifically incorporated into HIV-1 particles, and its uptake depended on a C-terminal region of the Gag precursor that includes NC.

Role of NC zinc fingers in the incorporation of Brox. HIV-1 NC contains two CCHC arrays that coordinate zinc and mediate specific interactions with the viral genomic RNA, leading to its encapsidation. Since the Bro1 domain-dependent uptake of ALIX into HIV-1 virions depends on the presence of at least one intact CCHC array in NC (37), we examined the importance of the CCHC arrays for the incorporation of Brox. To this end, we employed HIV-1 proviral constructs that cannot engage the Bro1 domain of ALIX because of in-frame deletions in NC that disrupt both of the CCHC arrays (37). Additionally, these constructs contain the Y_{36}s premature termination codon near the 3′ end of *gag*, which eliminates the ALIX binding site in p6 (37). Although HIV-1 NC plays a critical role in virus assembly, this function is thought to be mediated mainly by an N-terminal basic region (10, 39, 47). Furthermore, even in the absence of nearly all of NC, particle production could be restored by inactivating PR (34). We therefore used PR-negative proviral constructs to compensate for possible assembly defects. Furthermore, we transfected twice as much of the NC deletion mutants as of the parental proviral construct, based on the observation that Gag multimerization and membrane binding are highly concentration dependent (36).

The parental Y_{36}s proviral DNA and the NC deletion mutants were transfected into 293T cells along with a vector expressing HA-Brox, and viral particles released were analyzed for the incorporation of HA-Brox. As shown in Fig. 2A, VLP produced by the parental Y_{36}s construct incorporated HA-Brox (lane 2), indicating that the ALIX binding site in p6 was

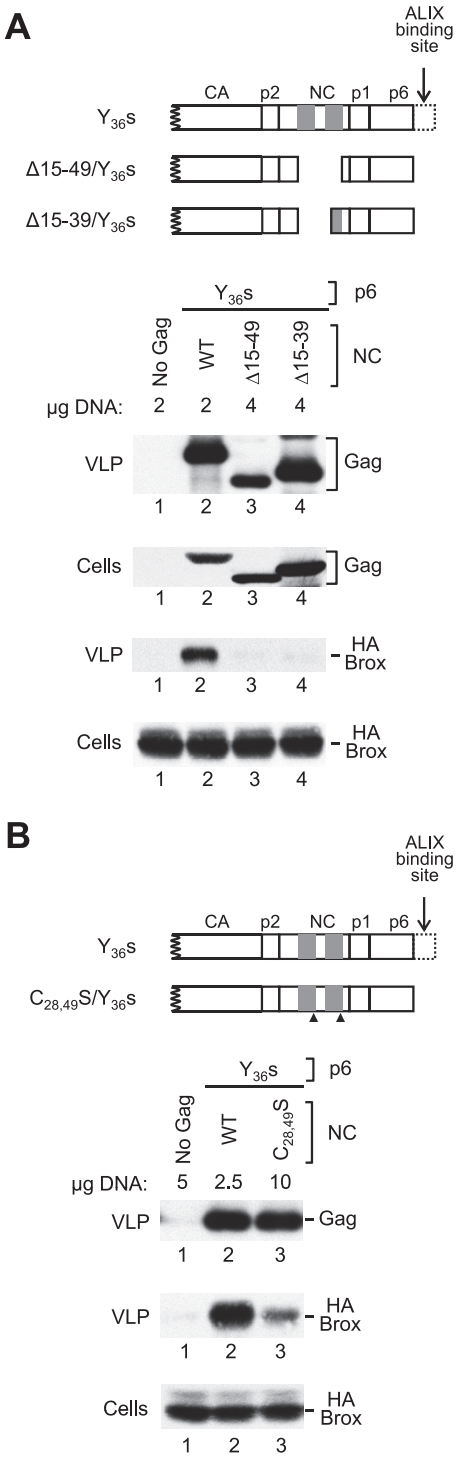

FIG. 2. The zinc fingers in NC are required for the incorporation of Brox. (A) Effects of deletions that remove all or part of the two zinc fingers, whose position within NC is indicated by shaded boxes. 293T cells were transfected with 2 μg of a vector expressing HA-Brox, together either with 2 μg of proviral DNAs expressing no Gag or WT Gag or with 4 μg of proviral DNAs expressing the indicated NC deletion mutants. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and HA-Brox as indicated. (B) Effect of disrupting both zinc fingers through point mutations. As indicated, 4 times more of the zinc finger mutant than of the parental provirus was transfected to compensate for a defect in particle production (37).

not required. In the same context, a deletion which precisely removed the two CCHC arrays and the intervening linker sequence from NC (Δ15–49) prevented the appearance of HA-Brox in the particulate fraction (lane 3). However, the Δ15–49 deletion also reduced VLP production to some extent. In contrast, a slightly less extensive deletion that removed the first CCHC motif and only a portion of the second (Δ15–39) did not compromise VLP production (Fig. 2A, lane 4), as previously reported (37). Nevertheless, only background levels of HA-Brox were detectable in the particulate fraction, indicating that the incorporation of Brox was prevented. We note that the Δ15–39 deletion also prevented the $LYPx_nL$-independent incorporation of ALIX, which occurs via the Bro1 domain (37). To determine whether NC needs to be able to coordinate zinc to mediate the incorporation of Brox, we employed the $C_{28,49S}$/Y_{36}s mutant (37), which has both CCHC arrays changed to CCHS. As shown in Fig. 2B, the $C_{28,49S}$ mutation in NC markedly reduced the incorporation of HA-Brox, which is similar to the effect of the mutation on the NC-mediated incorporation of ALIX (37). Together, these results indicate that NC associates with the Bro1 domain of ALIX and with Brox in similar fashions.

The ability to bind NC is a general property of Bro1 domains. Since the Bro1 domains of ALIX and Brox share less than 25% sequence identity, we considered the possibility that the association with HIV-1 NC involves a sequence or a structural element that is conserved among all Bro1 domains. To test this hypothesis, we examined whether NC can engage the Bro1 domains of HD-PTP and of RPH1. HD-PTP is a paralog of ALIX that has recently been shown to be required for endosomal cargo sorting and multivesicular body morphogenesis (14). The two remaining human Bro1 domain-containing proteins, RPH1 and RPH2, are scaffold proteins that are unrelated to ALIX, except in the Bro1 domain.

To examine whether HIV-1 NC can direct the uptake of the isolated Bro1 domains of HD-PTP or of RPH1 into viral particles, HA-tagged versions were coexpressed with HIV-1 proviral constructs expressing either no Gag, the WT Pr55 Gag polyprotein, or Z_{WT}-p6 Gag. The chimeric Z_{WT}-p6 Gag molecule is identical to WT Gag, except that the NC-p1 region is replaced by a foreign dimerization domain that rescues viral particle production (1). As shown in Fig. 3A, the Bro1 domains of HD-PTP and of RHP1 were both readily incorporated into particles formed by WT Pr55 but not into those formed by Z_{WT}-p6. Since the levels of particle formation were comparable, this result demonstrates that the uptake of the Bro1 domains required the presence of the NC-p1 region of Gag. Furthermore, their uptake depended at least in part on intact CCHC arrays within NC, since it was diminished if the CCHC arrays were disrupted by the $C_{28,49S}$ mutation (Fig. 3B).

To determine whether NC-p1 by itself can interact with Bro1 domains, GST-NCp1 was bound to glutathione-Sepharose beads and incubated with extracts from 293T cells expressing HA-tagged Brox, $RPH1_{Bro1}$, or $HD\text{-}PTP_{Bro1}$. All three epitope-tagged Bro1 domains were readily detected in association with GST-NCp1 but not with GST alone (Fig. 4). In the case of HA-Brox, we also examined its ability to associate with GST-NC and found that p1 did not contribute to the in vitro interaction (Fig. 4). Both GST-NC and GST-NCp1 pulled

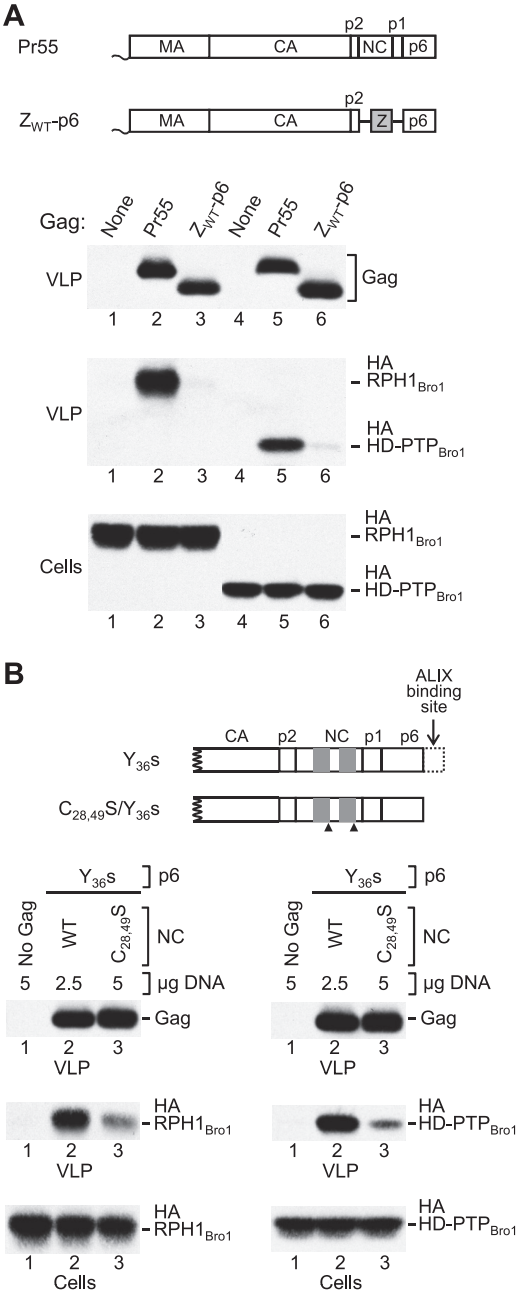

FIG. 3. Uptake of other Bro1 domains into VLP. (A) NC-mediated incorporation of the Bro1 domains of RHP1 and HD-PTP. 293T cells were transfected with proviral DNAs expressing no Gag, WT Gag, or Gag with a leucine zipper in place of NC-p1, together with vectors expressing the HA-tagged Bro1 domains of RHP1 or HD-PTP. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and HA-tagged proteins as indicated. (B) Effects of disrupting both zinc fingers in NC. Twice as much of the zinc finger mutant than of the parental provirus was transfected to compensate for a defect in particle production (37).

down two forms of HA-Brox that were separable by 10% SDS-PAGE. These likely corresponded to farnesylated and unmodified Brox, because the latter form is known to exhibit retarded migration in SDS-PAGE (23). NC did not discriminate between these species, because their relative abundances

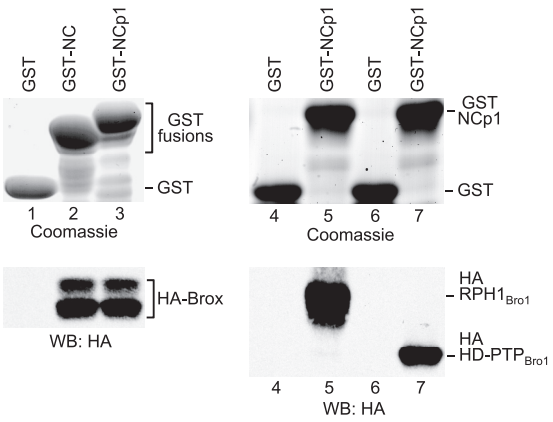

FIG. 4. In vitro interaction between NC and/or NCp1 and Bro1 domains. Glutathione-Sepharose beads decorated with bacterially expressed GST or GST fusion proteins were incubated with lysates from 293T cells expressing HA-tagged versions of Brox, RPH_{Bro1}, or $HD\text{-}PTP_{Bro1}$. GST proteins bound to the beads were detected by SDS-PAGE and Coomassie blue staining, and captured proteins were detected by Western blotting (WB) with anti-HA antibody.

in the input lysate and in the pulldown fractions were comparable (data not shown). Taken together, these results indicate that most, and probably all, human Bro1 domains share a feature that allows binding to NC.

Bro1 domains share the ability to stimulate the release of a minimal HIV-1 Gag protein. We previously described a minimal HIV-1 Gag molecule called Δ8–87/Δ126–277 (Fig. 5A) that produces VLP with WT efficiency (5) but is highly dependent on the presence of an intact ALIX binding site in p6 (41). For instance, VLP production was dramatically impaired by the $Y_{36}S$ mutation, which removes the entire ALIX binding site from p6 (41). Remarkably, VLP production by the $Y_{36}S$ version of the minimal Gag construct was potently enhanced by HA-$ALIX_{Bro1}$ (37). We therefore examined whether Brox shares the ability of the isolated ALIX Bro1 domain to stimulate VLP production in this system. Initial experiments were carried out with untagged Brox to examine the activity of the native protein. For comparison, we used pacsin 2, which harbors a curvature-sensing F-BAR domain that somewhat resembles the Bro1 domain in shape (31). As previously reported (37), HA-$ALIX_{Bro1}$ increased VLP production by the Δ8–87/Δ126–277/$Y_{36}S$ minimal Gag construct more than 10-fold without affecting Gag expression (Fig. 5B, lanes 1 and 2). Interestingly, the overexpression of native Brox led to a comparable increase in VLP production (Fig. 5B, lane 3). In contrast, HA-pacsin 2 had no effect (Fig. 5B, lane 4), even though its expression levels exceeded those of HA-$ALIX_{Bro1}$. An HA-tagged version of Brox and HA-$ALIX_{Bro1}$ were expressed at similar levels and exhibited a similarly pronounced activity in the minimal Gag release assay (Fig. 5C). However, as previously shown for HA-$ALIX_{Bro1}$ (37), HA-Brox did not augment virion production by WT HIV-1 (Fig. 5D). Also, HA-Brox had only a minor effect on the release of ΔPTAPP HIV-1, which lacks the primary L domain but is otherwise WT, and did not correct the characteristic Gag processing defect of this mutant (Fig. 5D). We and others previously showed that the ΔPTAPP mutant can be

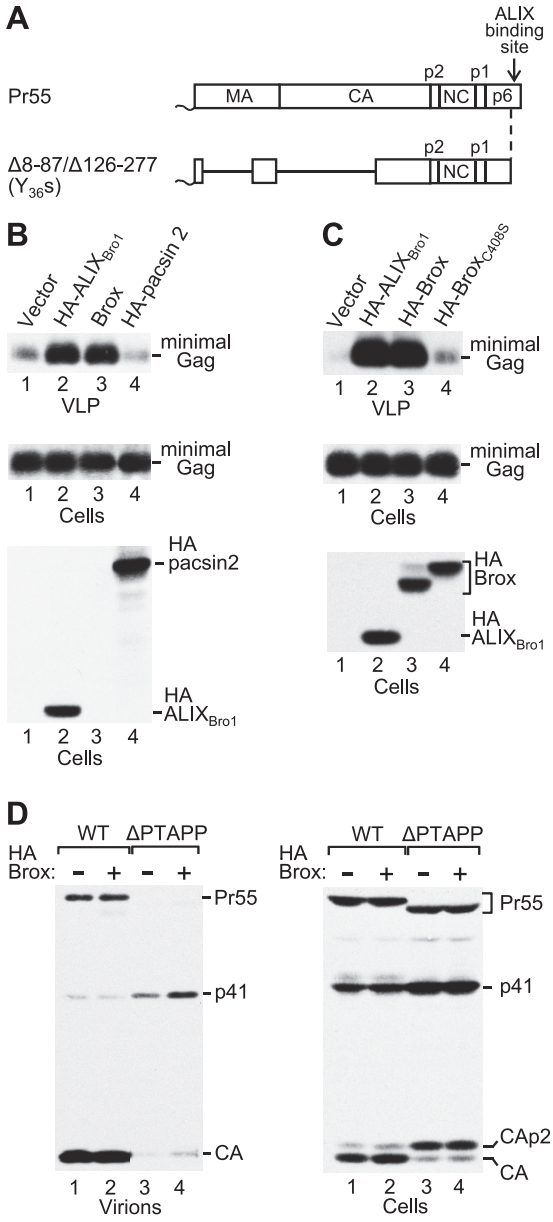

FIG. 5. Effect of Brox on particle production. (A) Schematic illustration of the Δ8–87/Δ126–277($Y_{36}S$) minimal Gag molecule, which lacks the globular domain of MA, the N-terminal domain of CA, and the binding site for the V domain of ALIX. (B) Rescue of VLP production by the Δ8–87/Δ126–277($Y_{36}S$) minimal HIV-1 Gag construct. 293T cells were transfected with 1 μg of proviral DNA, along with 2 μg of empty pBJ5 or versions expressing HA-$ALIX_{Bro1}$, untagged Brox, or HA-tagged pacsin 2. VLP production and the expression levels of Gag and HA-tagged proteins were examined by Western blotting with anti-CA and anti-HA antibodies. (C) Effect of HA-tagged WT or $C_{408}S$ Brox in the minimal Gag rescue assay, compared to that of HA-tagged $ALIX_{Bro1}$. (D) HA-Brox does not rescue ΔPTAPP HIV-1. 293T cells were transfected with 1 μg of WT or ΔPTAPP HXBH10, along with 2 μg of empty pBJ5 or a version expressing HA-Brox, and virion production and Gag expression levels were examined by Western blotting with anti-CA antibody 183-H12-5C.

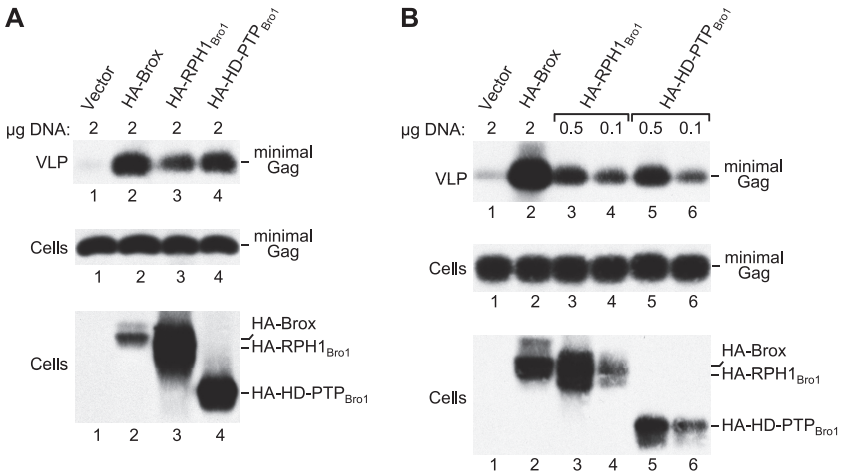

FIG. 6. Comparison of the effects of Brox and of the Bro1 domains of RPH1 and HD-PTP in the minimal Gag rescue assay. (A) Effects of equal amounts of the expression vectors. 293T cells were transfected with 1 μg of the Δ8–87/Δ126–277/Y_{36}s minimal Gag construct, along with 2 μg of empty pBJ5 or versions expressing HA-tagged Brox, $RPH1_{Bro1}$, or $HD\text{-}PTP_{Bro1}$. VLP pellets and the cell lysates were analyzed by Western blotting to detect Gag and HA-tagged proteins as indicated. (B) When expressed at comparable levels, Brox stimulates VLP production more potently than the Bro1 domains of RPH1 or HD-PTP.

potently rescued by full-length ALIX (16, 43) but not by HA-$ALIX_{Bro1}$ (37).

The comparable activities of $ALIX_{Bro1}$ and of Brox in the minimal Gag release assay raised the possibility that this activity is shared by Bro1 domains in general. To test this hypothesis, we examined whether the Bro1 domains of RPH1 and of HD-PTP also affect the release of Δ8–87/Δ126–277/Y_{36}s Gag. As shown in Fig. 6A, both HA-$RPH1_{Bro1}$ and HA-$HD\text{-}PTP_{Bro1}$ were capable of enhancing the release of the minimal Gag molecule. Quantitation indicated that HA-$RPH1_{Bro1}$ and HA-$HD\text{-}PTP_{Bro1}$ increased VLP release by 16- and 24-fold, respectively. By comparison, HA-Brox had a 31-fold effect in this experiment, even though the expression levels of HA-Brox were considerably lower than those of HA-$RPH1_{Bro1}$ and HA-$HD\text{-}PTP_{Bro1}$ (Fig. 6A). To obtain more comparable expression levels, the vectors encoding HA-$RPH1_{Bro1}$ and HA-$HD\text{-}PTP_{Bro1}$ were titrated down (Fig. 6B). This experiment confirmed that HA-Brox is more potent than HA-$HD\text{-}PTP_{Bro1}$ in promoting the release of Δ8–87/Δ126–277/Y_{36}s Gag and that HA-$HD\text{-}PTP_{Bro1}$ in turn is somewhat more potent than HA-$RPH1_{Bro1}$. Nevertheless, even at rather low expression levels, HA-$HD\text{-}PTP_{Bro1}$ and HA-$RPH1_{Bro1}$ both continued to show activity compared to the vector control (Fig. 6B, compare lanes 1, 4, and 6).

Role of CHMP4 binding. Ichioka et al. recently demonstrated that Brox interacts with CHMP4B, indicating that it possesses an authentic Bro1 domain (23). Because CHMP4 binding site mutants of full-length ALIX fail to rescue HIV-1 L domain mutants (16, 43), a role for CHMP4 in the stimulation of VLP production by Brox appeared to be possible. Previous work showed that mutations in an exposed hydrophobic patch on the Bro1 domain of human ALIX (I_{212}D and L_{216}D) eliminate binding to CHMP4 proteins (16, 43). We therefore made the equivalent mutations in Brox (L_{208}D and L_{212}D) and tested their effects on CHMP4B binding in a coprecipitation assay. To this end, cells expressing WT or mutant versions of HA-Brox together with FLAG-tagged CHMP4B were lysed in 0.5% NP-40 buffer, and proteins precipitated from the lysates with anti-HA antibody were analyzed by Western blotting. We found that CHMP4B-FLAG coprecipitated specifically with HA-Brox, as expected (Fig. 7A, lanes 1 and 2), and that the L_{208}D and L_{212}D mutations both eliminated the Brox-CHMP4B interaction (lanes 3 and 4). In contrast, the C_{408}S mutation, which was previously shown to prevent the farnesylation of Brox (23), had no effect on the in vivo interaction (lane 5). As previously reported, the C_{408}S mutation altered the electrophoretic mobility of Brox (23), and the mutant comigrated with a minor, presumably unmodified species of WT Brox (Fig. 5C and 7A).

We also examined the effects of the mutations on the incorporation of Brox into viral particles formed by WT HIV-1 Gag. As shown in Fig. 7B, the L_{208}D and L_{212}D mutations both reduced the uptake of HA-Brox two- to threefold (lanes 3 and 4). On 10% SDS-polyacrylamide gels, which allowed a clear separation of farnesylated from unmodified Brox, it also became apparent that the farnesylated form of WT Brox was selectively incorporated (Fig. 7B, lane 2). Consistent with this observation, the C_{408}S mutation decreased the incorporation of HA-Brox into HIV-1 particles to near background levels (Fig. 7B, lane 5).

Lastly, we examined the ability of the Brox mutants to stimulate VLP production by the Δ8–87/Δ126–277/Y_{36}s minimal Gag construct. We found that the L_{208}D and L_{212}D mutations both reduced the activity of HA-Brox in this assay (Fig. 7 C, lanes 3 and 4). Nevertheless, in spite of the effects of the L_{208}D and L_{212}D mutations on the Brox-CHMP4B interaction, both mutants clearly retained a significant level of activity. Specifically, quantitation revealed that both mutants enhanced VLP production about 12-fold in the experiment shown in Fig. 7C. In contrast, the C_{408}S farnesylation site mutant exhibited no or only marginal activity in repeated experiments (Fig. 7C, lane 5; also see Fig. 5C, lane 4). Taken together, these results raise the possibility that the

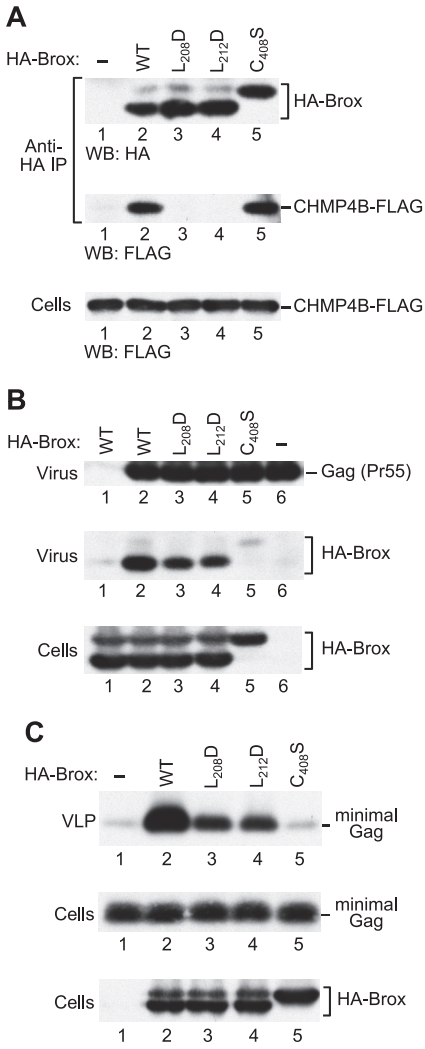

FIG. 7. Role of CHMP4 binding in the effect of Brox in the minimal Gag rescue assay. (A) Effects of point mutations in Brox on the interaction with CHMP4B. 293T cells were transfected with vectors expressing WT HA-Brox or the indicated mutants together with CHMP4B-FLAG. Proteins immunoprecipitated (IP) with anti-HA antibody and the cell lysates were analyzed by Western blotting (WB) as indicated. (B) Effects of point mutations in Brox on its incorporation into HIV-1 virions. 293T cells were transfected with proviral DNA encoding no Gag (lane 1) or WT Gag (lanes 2 to 6), along with WT or mutant HA-Brox, or empty vector, as indicated. Virion pellets and the cell lysates were analyzed by Western blotting to detect Gag and HA-Brox as indicated. (C) Farnesylation is essential for the activity of Brox in the minimal Gag rescue assay, whereas CHMP4 binding is not. The experiment was performed as described for Fig. 5B.

interaction with CHMP4B is not essential for the ability of Brox to enhance VLP production.

DISCUSSION

The human Bro1 domain proteins include ALIX, HD-PTP, Brox, and the closely related RHP1 and RHP2 proteins. HIV-1 engages ALIX through its $LYPx_nL$-type L domain in p6, which serves as a docking site for the central V domain of ALIX (17). Recently, it emerged that ALIX additionally interacts with the NC region of HIV-1 Gag through its N-terminal Bro1 domain (37). We now report that Brox, a Bro1 domain-only protein, is also capable of interacting with HIV-1 NC, as are the isolated Bro1 domains of HD-PTP and of RPH1. While HD-PTP is a paralog of ALIX, RHP1 has a different domain organization. Also, the Bro1 domains of Brox and RHP1 exhibit only limited sequence homology to that of ALIX. Our results thus reveal that HIV-1 NC can interact with widely divergent Bro1 domains.

The ALIX-NC interaction depends on conserved zinc finger motifs in NC (37), which are thought to mediate sequence-specific interactions with the packaging signal in the genomic viral RNA (2, 11). Nevertheless, nucleic acid does not appear to be required for the ALIX-NC interaction, because the in vitro interaction was insensitive to nuclease treatment (37). The present study indicates that the zinc fingers in NC have a general affinity for Bro1 domains, since the disruption of both zinc fingers by the $C_{28,49S}$ mutation interfered with the incorporation of Brox and of the isolated Bro1 domains of RHP1 and HD-PTP into VLP. Similarly, the $C_{28,49S}$ mutation interferes with the binding of ALIX to NC (37). Of note, in a WT HIV-1 context, the $C_{28,49S}$ mutation caused a defect in viral particle production and a Gag processing defect that resembled that of HIV-1 L domain mutants (12, 37). On the other hand, the $C_{28,49S}$ mutation did not further reduce particle production in the context of an HIV-1 L domain mutant, which suggested that the NC zinc fingers and the L domain are required for the same step in virus assembly (37).

The membrane fission defect of HIV-1 mutants that lack the P(T/S)AP motif required for Tsg101 binding can be fully corrected by overexpressing ALIX (16, 43). In contrast, the isolated Bro1 domain of ALIX was inactive in the ΔPTAP rescue assay (37), as expected since both the V domain and the C-terminal proline-rich domain of ALIX are required for its ability to rescue Tsg101 binding site mutants (6, 16, 43). Similarly, in the present study, the overexpression of the Bro1 domain-only protein Brox did not significantly enhance VLP production by ΔPTAP HIV-1. However, we found that Brox is as potent as the isolated ALIX Bro1 domain in rescuing a minimal Gag construct that retains the PTAP L domain but lacks the binding site for the V domain of ALIX. Furthermore, the isolated Bro1 domains of HD-PTP and of RPH1 also exhibited activity in this assay. Apart from the ALIX binding site in p6, the minimal Gag construct used in these experiments lacked all Gag-Gag interaction sites in the N-terminal half of the Gag precursor and thus was presumably attenuated in its ability to bend the plasma membrane away from the cytosol. One possible interpretation of our results is that isolated Bro1 domains, although unable to trigger the membrane fission event required for viral particle release, can nevertheless assist Gag in the deformation of cellular membranes.

The observation that all Bro1 domains tested were active in the minimal Gag release assay pointed to the involvement of a feature that is shared among widely divergent Bro1 domains. One such feature appears to be an interaction site for CHMP4 family members (26). For instance, the Bro1 domains of HD-PTP and of Brox both interact with CHMP4B (23, 24), and binding to CHMP4 has been shown to be essential for biological function in the case of HD-PTP (14). Although RPH2 did not interact with human CHMP4 proteins in one study (35),

recent results obtained with ALIX raise the possibility that the docking site for CHMP4 can be occluded by autoinhibition (48). Surprisingly, we found that point mutations in Brox that prevented its interaction with CHMP4B also reduced its NC-dependent uptake into HIV-1 particles. Since the steady-state levels of Brox were unaffected, this observation raises the possibility that the CHMP4 binding site contributed to the interaction with Gag. Interestingly, Brox mutants that showed no interaction with CHMP4B nevertheless retained considerable activity in the minimal Gag rescue assay, indicating that this activity may be intrinsic to the Bro1 domain itself. However, we cannot exclude that these Brox mutants retained some ability to bind CHMP4 in vivo, even though no binding was evident in our coprecipitation assay.

The Bro1 domain has the shape of a boomerang, which could theoretically generate negative curvature by interacting with the cytosolic face of cellular membranes through its convex surface (16, 26). Consistent with this possibility, the convex face of the Bro1 domain of a yeast homolog of ALIX possesses a highly basic patch that could interact with anionic phospholipids (26), and this basic patch is to some extent conserved in ALIX (16). Furthermore, ALIX binds to bilayers that contain the unconventional phospholipid lysobisphosphatidic acid, which is thought to regulate the formation of invaginations with negative curvature at the limiting membrane of late endosomes (15, 30).

An involvement of cellular proteins in the generation of membrane curvature by Gag is suggested by reports showing that the PPXY-type L domains of human T-cell leukemia virus type 1 and of Mason-Pfizer monkey virus are required early during bud formation (20, 27). More specifically, there is evidence that ALIX plays a role in membrane deformation by equine infectious anemia virus Gag, because budding was arrested at an early stage in the presence of dominant-negative ALIX (41). In light of this observation, we propose that widely divergent Bro1 domains stimulate VLP production by a partially defective minimal Gag molecule because they share the capacity to interact with HIV-1 Gag and to generate negative membrane curvature.

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