An Alix Fragment Potently Inhibits HIV-1 Budding

CHARACTERIZATION OF BINDING TO RETROVIRAL YPXL LATE DOMAINS

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The retroviral structural protein, Gag, contains small peptide motifs known as late domains that promote efficient virus release from the infected cell. In addition to the well characterized PTAP late domain, the p6 region of HIV-1 Gag contains a binding site for the host cell protein Alix. To better understand the functional role of the Gag/Alix interaction, we overexpressed an Alix fragment composed of residues 364–716 (Alix 364–716) and examined the effect on release of wild type (WT) and Alix binding site mutant HIV-1. We observed that Alix 364–716 expression significantly inhibited WT virus release and Gag processing and that mutation of the Alix binding site largely relieved this inhibition. Furthermore, Alix 364–716 expression induced a severe defect on WT but not mutant particle morphology. Intriguingly, the impact of Alix 364–716 expression on HIV-1 release and Gag processing was markedly different from that induced by mutation of the Alix binding site in p6. The association of Alix 364–716 with HIV-1 and equine infectious anemia virus late domains was quantitatively evaluated by isothermal titration calorimetry and surface plasmon resonance techniques, and the effects of mutations in these viral sequences on Alix 364–716 binding was determined. This study identifies a novel Alix-derived dominant negative inhibitor of HIV-1 release and Gag processing and provides quantitative information on the interaction between Alix and viral late domains.

An essential phase of the retroviral replication cycle involves the budding and release of nascent virus particles from the infected host cell. The budding process requires the trafficking of the Gag polyprotein precursor from the cytosol to the plasma membrane, currently thought to be the principal site of particle assembly in most cell types. During or shortly after virus budding and release, the Gag precursor protein is cleaved by the viral protease into the mature Gag proteins: matrix, capsid, and nucleocapsid. In the case of HIV-1, an additional domain, p6, is located at the C terminus of the Gag precursor. The protease-mediated cleavage of the Gag precursor is necessary for the formation of an infectious virus particle (reviewed in Ref. 1).

Retrovirus budding requires that the virus co-opt the host cell endosomal sorting machinery. This is achieved through an interaction between a motif in Gag known as the late or L domain and a component of the cellular protein trafficking machinery (2–4) Three retroviral L domains have been characterized thus far: P(T/S)AP, PPPY, and YPDL. P(T/S)AP is located within the p6 domain of HIV-1 Gag and has been shown to promote release via an interaction with Tsg101, a component of the cellular ESCRT-1 protein complex (5–10). Binding between the P(S/T)AP motif of p6 and Tsg101 involves the N-terminal, ubiquitin E25 variant domain of Tsg101 (11). The PPPY-type L domain, found in the Gag proteins of retroviruses, such as murine leukemia virus (12), is thought to promote release via an interaction with Nedd4-like ubiquitin ligases (13–15). Finally, EIAV contains a YPDL late domain that promotes release via an interaction with the human ortholog of the yeast protein Bro1, known as Alix or AIP1 (16–19).

Some retroviruses contain more than one late domain within their respective Gag proteins (3, 4). For example, human T-cell leukemia virus type 1 contains the bipartite PPPYVEPTAP late domain (20, 21). Interestingly, two groups have identified a region C-terminal to the well characterized P(T/S)AP late domain in HIV-1 Gag that is capable of binding Alix in vitro (18, 19). Mutational studies of the Alix binding site indicate that whereas point mutations within a minimal Gag context reduce virus production 10-fold (18), point mutations in the Alix binding site within full-length Gag (22) or deletion of the binding site (23, 24) have little or no effect on virus production.

As mentioned above, retrovirus budding depends on the late domain-mediated hijacking of the cellular endosomal sorting machinery. The physiological role of this cellular machinery is
to regulate the biogenesis of vesicles that bud inwardly from the limiting membrane of late endosomes to form structures known as multivesicular bodies (MVBs) (25). Throughout all eukaryotes, the MVB pathway plays a central role in the physiological degradation of transmembrane proteins. This pathway packages transmembrane proteins destined for degradation into MVBs that subsequently fuse with the lysosome (26). The pathway is largely conserved in yeast, where it is used not only to direct transmembrane proteins to the vacuole for degradation but also to deliver newly synthesized lysosomal or vacuolar hydrolases to the vacuole (26). Indeed, the core MVB machinery was first revealed through genetic analysis of protein sorting to the yeast vacuole (27). Genetic defects in this pathway manifest as a class E vacuolar protein sorting (VPS) phenotype, characterized by the formation of an aberrant compartment known as the class E compartment adjacent to the vacuole. The MVB pathway is used in more specialized cell types for antigen presentation and exosome production, in which cargo is delivered to the cell surface rather than to the lysosome. Viral budding appears to be topologically similar to the exosome pathway (3, 4).

The class E VPS genes encode the subunits of three endosomal sorting complexes, ESCRT-I (Vps23, Vps28, and Vps37) (28), ESCRT-II (Vps22, Vps25, and Vps36) (29), and ESCRT-III (Vps2, Vps20, Vps24, Vps60, Did2, and Snf7) (30), and the associated proteins Rsp5 (31), Vps4 (32), Vps27 (33, 34), and Bro1 (35). The human ESCRT-III complex and Vps4 are required for the budding of all viruses known to use the MV pathway (3, 4) and appear to constitute the core machinery for vesicle invagination and budding. The remaining proteins and complexes are primarily responsible for the recruitment of specific cargo into the MVB pathway. Vps27, ESCRT-I, and ESCRT-II participate in the recruitment of monoubiquitinated proteins. Of these three, only ESCRT-I has been clearly shown to play a role in viral budding. As mentioned above, YPXL late domains and similar motifs in cellular proteins are recruited into the MVB pathway via their interaction with Alix (16, 18, 19, 36).

Alix is a multidomain adaptor protein that contains the N-terminal Bro1 domain responsible for ESCRT-III binding and endosomal recruitment (37), a central domain required for binding to the late domain of EIAV (16), and a C-terminal proline-rich domain that interacts with Tsg101 (18, 19) and a variety of proteins involved in endocytosis (38–40) and apoptosis (39, 41–43).

Many of the interactions in the MV pathway have been quantitated, and the structures of a number of the relevant complexes have been determined (44). Despite the importance of the YPXL motif/Alix interaction in EIAV budding, this interaction has not been quantitated, and the determinants for binding are not well understood. The motif appears to be bipartite, containing both an N-terminal YPXL sequence and a C-terminal LXXLL sequence (Fig. 1). The relative contributions of each portion of the motif in different viruses are unclear. We therefore isolated the YPXL-binding domain of Alix (Alix 364–716) and quantitatively measured its binding to HIV-1 and EIAV late domains. We also examined the effect of Alix 364–716 expression on virus release and observed that this protein fragment acts as a potent dominant negative inhibitor of HIV-1 budding.

The Viral Late Motif Binding Domain of Alix

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sis kits (Stratagene). All mutations were confirmed by DNA sequencing. GST-Alix 364–716, GST, GST-HIV-1 p6, and GST-EIAV p9 were expressed in E. coli BL21(DE3). Cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside and grown at 20 °C overnight. Harvested cells were lysed in 1× phosphate-buffered saline (PBS) (pH 7.4), and the supernatant was applied to a glutathione-Sepharose column (Amersham Biosciences). Tobacco etch virus protease was used to cleave the GST fusion protein, and tobacco etch virus protease was removed by passing through TALON resin (BD Biosciences). Protein was concentrated and applied to a Superdex 200 column (Amersham Biosciences) in 10 mM HEPES (pH 7.4), 150 mM NaCl, and 2 mM dithiothreitol.

**Cell Culture, Transient Transfections, and Metabolic Labeling**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum (HyClone), 1× glutamine, and 1× penicillin/streptomycin at 37 °C. Cells were plated overnight at a density of 1.0 × 10^6 cells/well of a 6-well cell culture plate for virus release and electron microscopy (EM) experiments. Cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol. To measure HIV-1 release efficiency, cells were labeled with [35S]Met/Cys protein labeling mix (PerkinElmer Life Sciences) for 2.5 h in Met/25°C. For studies of binding to full-length HIV-1 p6, WT or mutant HIV-1 p6 and EIAV p9 was measured simultaneously by passing these proteins over flow cells coupled to GST or GST-Alix 364–716, with association and dissociation times of 100 and 300 s, respectively. Between injections of p6 and p9 proteins, surfaces were regenerated with an injection of Hepes-buffered saline supplemented with 500 mM NaCl for 15 s at 100 μl min^-1.

**Viruses and Gag Processing Assays**—Virus release assays were performed as described previously (46). Briefly, transfected HeLa cells were metabolically labeled with [35S]Met/Cys, and virions were pelleted by ultracentrifugation. Cell and virus lysates were immunoprecipitated with HIV immunoglobulin (HIV-Ig) obtained from NABI and the NHLBI, National Institutes of Health (NIH), through the NIH AIDS Research and Reference Reagent Program and separated by SDS-PAGE followed by fluorography. Expression of virally encoded proteins was quantified by PhosphorImager analysis. Virus release efficiency relative to the absence of Alix 364–716 = virus release efficiency in the presence of Alix 364–716/virus release efficiency in the absence of Alix 364–716, where virus release efficiency = virus-associated Gag/virus-associated Gag + cell-associated Gag. p55/(p24 + p25) relative to the absence of Alix 364–716 = (p55/(p24 + p25)) in the presence of Alix 364–716/(p55/(p24 + p25)) in the absence of Alix 364–716, where (p55/(p24 + p25)) = cell-associated p55/cell-associated p24/p25). For detection of Alix 364–716 expression, cell lysates were separated on SDS-PAGE and subsequently transferred to polyvinylidene difluoride membrane. Western blotting was performed using rabbit anti-Alix polyclonal antiserum (kindly provided by W. Sundquist). The Alix fragment bands were captured using the Alpha Innotec FluorTec SP chemiluminescence imager, and the resulting data were quantified using the AlphaEaseFC quantification module. Analysis of EIAV particle release was performed essentially as described above, except that lysates were immunoprecipitated with horse anti-EIAV antiserum (kindly provided by R. Montelaro).

**Electron Microscopy**—Cells were transfected for EM as for the HIV-1 virus release assay. Cells were washed with Dulbecco’s PBS and fixed for EM analysis 30 h post-transfection as described previously (46). Cells were examined by a Hitachi H7000 electron microscope.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was conducted as described previously (47, 48). Briefly, 6 × 10⁴ HeLa cells were plated in Nalgé Nunc slides overnight at 37 °C and transfected as described above. 24 h posttransfection, cells were washed with PBS and subsequently fixed for 30 min to 1 h at room temperature in PBS containing 3.7% formaldehyde. After fixation, cells were washed four or five times with PBS, permeabilized with 0.1% Triton X-100 in PBS, quenched with 0.1 M glycine in PBS, and incubated with primary antibody for 1 h. Cells were then washed four times with PBS, incubated with secondary antibody, and washed three times with PBS, and then the coverslip was mounted using Aqua Poly Mount mounting medium (Polysciences, Inc). Cells were then examined using the softWoRx visualization module on a DeltaVision RT microscope.

**Surface Plasmon Resonance**—The binding of Alix 364–716 to WT and mutant HIV-1 p6 and EIAV p9 was measured with a Biacore T100 system at 25 °C with a flow rate of 20 μl min^-1. The CM5 chip was activated using 1:1 N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at a flow rate of 5 μl min^-1 for 20 min. GST (30 μM) and GST-Alix 364–716 (30 μM) in 10 mM acetate buffer (pH 4.0, 5.0, and 6.0) were passed over separate flow cells at 5 μl min^-1 for 40 min, and this was followed by a blocking step using ethanolamine (1 M, pH 8.5) at 5 μl min^-1 for 20 min. All binding experiments were performed in Hepes-buffered saline buffer. Binding of Alix 364–716 to p6 and p9 was measured simultaneously by passing these proteins over flow cells coupled to GST or GST-Alix 364–716, with association and dissociation times of 100 and 300 s, respectively. Between injections of p6 and p9 proteins, surfaces were regenerated with an injection of Hepes-buffered saline supplemented with 500 mM NaCl for 15 s at 100 μl min^-1.

**Isothermal Titration Calorimetry**—The peptides DKELYPLTLSRLSFLG (HIV-1 p6–16) and QTQNYLPLESK (EIAV p9–15) were obtained from New England Peptide at >99% purity. Alix 364–716 was dialyzed into 10 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM dithiothreitol and was used at a concentration of 20 μM. Peptides were dissolved in the same buffer and were used at 400 μM. Titration calorimetry measurements were taken using a MicroCal VP-ITC instrument at 25 °C. For studies of binding to full-length HIV-1 p6, WT or mutant proteins were dialyzed into 10 mM HEPES (pH 7.5), 150 mM NaCl, and 5 mM dithiothreitol. Alix 364–716 and p6 were used at concentrations of 20 and 400 μM, respectively. The concentrations of HIV-1 p6 Y36A, Y36F, and LYP (35–37) to AAA mutants were obtained by using the BCA protein assay kit (Pierce), and other concentrations were obtained by UV absorption spectroscopy at 280 nm. Peptides and full-length HIV-1 p6 samples were injected into 1.8 ml of Alix in 25 aliquots of 10 μl each at 240-s intervals. Data obtained from peptide injections into 1.8-ml buffer blanks were subtracted from the experimental data before analysis using Origin (Microcal). Affinities reported are the mean ± S.D. determined from three independent measurements.
The data indicated that overexpression of Alix 364–716 strongly inhibited the release of WT HIV-1, with an ~5-fold reduction in particle production upon fragment overexpression (Fig. 2, A and C). The fragment had significantly less effect on the production of L41A and L41R mutant virions (Fig. 2, A and C), suggesting that the effect of the fragment on particle production was due to its direct binding to Gag. Levels of Alix 364–716 expression were comparable between samples (Fig. 2B). We also observed that Alix 364–716 overexpression severely disrupted Gag processing, resulting in an increase in the ratio of Pr55Gag to p25/p24 (Fig. 2, A and D). Again, the effect of Alix 364–716 on Pr55Gag processing was mitigated by the Alix binding site mutations L41A and L41R (Fig. 2, A and D). Overexpression of Alix 364–716 also led to a consistent reduction in the cell-associated levels of WT but not L41A or L41R Gag (Fig. 2A). Interestingly, in contrast to the effects of Alix 364–716 expression on WT virus release and processing of Pr55Gag to p25/p24, the L41A and L41R mutants in the absence of Alix 364–716 displayed WT levels of release but elevated levels of the Gag processing intermediates p41 and p25 (Fig. 2A).

To further examine the effect of Alix 364–716 expression on retroviral particle release, a WT EIAV Gag expression construct (49) was transfected into HeLa cells in the presence or absence of the Alix 364–716 expression vector. EIAV VLP production was severely inhibited by Alix 364–716 expression (Fig. 2E). These observations support the results presented in Fig. 2 and confirm that binding of Alix 364–716 to Gag strongly inhibits retrovirus particle production.

**RESULTS**

**Overexpression of Alix 364–716 Interferes with HIV-1 Particle Production**—Based on the findings that the central region of Alix binds the EIAV YPDL late domain (16) and that the p6 region of HIV-1 Gag contains an Alix binding site (16, 18, 19), we examined whether overexpression of Alix 364–716 would interfere with HIV-1 release. As controls, we measured the effect of Alix 364–716 expression on the release of HIV-1 mutants containing substitutions (L41A and L41R) in the Alix binding site.
condensed core (Fig. 3A). Expression of Alix 364–716 in cells transfected with the WT HIV-1 molecular clone led to a drastic change in particle morphology. Specifically, both budding and released particles displayed a multibudded phenotype, with numerous particles tethered to the plasma membrane (Fig. 3B).

In contrast, Alix 364–716 expression in cells transfected with the L41A or L41R mutants had little or no effect on particle morphology (Fig. 3, compare C and E with D and F, respectively).

To quantify the effect of Alix 364–716 on the morphology of budding particles, we scored the ratio of budding (not released) structures to budded (released) particles. In samples transfected with WT pNL4-3, the ratio of budding to released particles was 0.9. In the presence of Alix 364–716, this ratio increased markedly to 2.2. For the L41A and L41R mutants in the absence of Alix fragment, the ratio of budding to released particles was 1.3. In the presence of Alix 364–716, this ratio was 1.5 and 0.7 for L41A and L41R, respectively. These data confirm that the Alix fragment markedly impaired the budding of WT HIV-1 virions but had little or no effect on particle budding for the Alix binding-deficient L41A and L41R mutants.

Alix 364–716 Overexpression Does Not Induce the Formation of an Aberrant Endosomal Compartment—Previous studies indicated that overexpression of full-length Tsg101 inhibits HIV-1 release by directly binding to the PTAP late domain in p6, in the absence of any overt disruption of the cellular endosomal sorting machinery (5, 7). To determine whether Alix 364–716 inhibits virus release through the formation of a mammalian class E compartment similar to that induced by overexpression of Tsg101, the localization of Alix 364–716 was examined by immunofluorescence. HeLa cells were transfected with the vector expressing an N-terminally hemagglutinin-tagged Alix 364–716, full-length Tsg101 (TSG-F) (7), or TSG-5′ (5). 24 h post-transfection, cells were processed for immunostaining as described under "Experimental Procedures."
that Alix 364–716 displays a diffuse cytosolic staining pattern similar to that of TSG-5 but distinct from that of TSG-F (Fig. 4). The diffuse cytosolic localization pattern of Alix 364–716 suggests that expression of this fragment does not inhibit virus release through the formation of swollen aberrant endosomes. These results reinforce the conclusion from the data presented in Figs. 2 and 3 that inhibition of WT release and Gag processing mediated by Alix 364–716 is due to binding of the fragment to Gag.

Alix 364–716 Binds HIV-1 p6 and EIAV p9—To characterize the biophysical and biochemical properties of Alix 364–716 in vitro, this region was expressed as a recombinant protein in E. coli and purified to homogeneity. This recombinant Alix 364–716 fragment is predominantly monomeric on gel filtration chromatography, although a small fraction of the material migrates at a higher apparent molecular mass (Fig. 5).

Alix 364–716 binds directly to HIV-1 p6 and EIAV p9 as judged by both SPR and ITC. Alix 364–716 was immobilized and p6 and p9 proteins were the analyte in the SPR experiments. Robust binding to EIAV p9 with $K_d = 8 \mu M$ was observed (Fig. 6). HIV-1 p6 also bound to Alix 364–716 in these experiments. The p6 protein binding curve showed a decrease in resonance response at the highest concentrations tested (250 and 500 $\mu M$) (data not shown), suggesting that the p6 protein was aggregating at high concentrations. Binding was not saturated at the highest concentration (100 $\mu M$) at which there was no sign of aggregation. It was therefore not possible to rigorously fit a binding curve to the data, but the results suggested that the binding of HIV-1 p6 to immobilized Alix 364–716 was $\sim 100 \mu M$.

The binding of HIV-1 p6 to Alix 364–716 in solution was characterized by ITC. The two proteins interact with $K_d = 8 \mu M$ and unit stoichiometry (Fig. 7). The elevated affinity of p6 for Alix 364–716 when measured by ITC as compared with SPR is probably an artifact of the aggregation of HIV-1 p6 under the conditions of the SPR study. The consistency of the ITC results for HIV-1 p6 and EIAV p9 and the consistency between the EIAV p9 SPR and ITC results argue that the ITC data reliably mirror the true affinities of these proteins for Alix 364–716.

To determine whether the YPXL peptide motif conserved between HIV-1 p6 and EIAV p9 was responsible for Alix 364–716 binding, peptides were synthesized corresponding to the YPXL motifs from these two proteins. The HIV-1 p6-derived peptide DKELYPLTSLRSLFGN (HIV-1 p6–16) yielded a complex curve for binding to Alix 364–716 that required fitting with a two-site model. The best fit revealed a single high affinity binding site with $n = 0.7$ and $K_d = 4.7 \mu M$ (Table 1 and data not shown). The $K_d$ value is not significantly different from the value of 8 $\mu M$ obtained for full-length p6. The second binding mode suggested by the fit has $n = 13$ and $K_d = 0.5 \text{ mM}$ (data not shown), which we interpret to reflect nonspecific precipitation of this hydrophobic peptide onto the surface of Alix 364–716 at very high concentrations. The EIAV p9-derived peptide QTQNLYPDLEIKKE (EIAV p9–15) yielded a binding curve similar to that for the HIV-1 p6–16 peptide and was best fit with a two-site model. The best fit yielded $n = 0.7$ and $K_d = 5.9 \mu M$ for the high affinity binding site, and $n = 0.3$ and $K_d = 1 \text{ mM}$ for the putative nonspecific binding mode (Table 1 and data not shown).
**Sequence Determinants for Late Domain Binding to Alix 364–716**—To determine which late domain residues dictate specificity for binding to Alix 364–716, a series of mutants was generated in the full-length HIV-1 p6 and EIAV p9 proteins. The p6 point mutants E34A, L35A, Y36A, Y36F, P37A, L41A, L41R, and L44A were constructed to test the roles of these individual side chains in recognition. The triple mutant L35A/Y36A/P37A was made to test the role of the LYP portion of the motif, and the deletion mutant Δ34–35 was suggested by the presence of this mutation in an HIV-1 isolate from a long term nonprogressor (50). The binding of these mutants was characterized by ITC (Fig. 7 and Table 1). The point mutations Y36A, L41R, and Δ34–35 deletion mutants reduce binding 20-fold or more, to near the detection limit of the ITC assay. The triple LYP mutation also completely abrogates binding. The P37A, L41R, and Δ34–35 deletion mutants reduce binding 20-fold or more, to near the detection limit. In contrast, mutations E34A, L35A, and Y36F are inconsequential to binding. We conclude that the side chains of Pro37, Leu41, and Leu35 are critical binding determinants. The presence of an aromatic, or at least bulky, side chain at Tyr36 is critical, but the Tyr hydroxyl group is not required. The Δ34–35 deletion appears to disrupt the structural integrity of the binding site, since it abrogates binding, although the individual side chains of Glu34 and Leu35 are not essential.

The conserved residues of the EIAV p9 YPXL motif were mutated to compare the binding determinants in the two viruses. Binding to these mutants was assessed by SPR, since we observed that EIAV p9, in contrast to HIV-1 p6, binds robustly to immobilized Alix 364–716. Mutants L22A, Y23A, Y23F, P24A, L26A, L26R, I29A, and L22A/Y23A/P24A were constructed and purified, and their binding was analyzed (Fig. 6 and Table 1). None of the EIAV p9 mutants bind appreciably to Alix 364–716, with the exception of Y23F. This latter mutant displays essentially WT binding, consistent with the finding for the p6 Y36F mutant. The pattern of mutational results is thus nearly identical for the YPXL motifs in HIV-1 p6 and EIAV p9. The only major difference is that the p9 L22A mutation completely abrogates binding, whereas the p6 counterpart, L35A, reduces binding by less than 2-fold.

**DISCUSSION**

Although several studies have indicated that the YPXL motif located in the p9 domain of EIAV Gag serves as a late domain by interacting with the host cell protein Alix (9, 16, 18, 19), the role of the YPXL motif in HIV-1 Gag function is not well understood. Studies from other laboratories have demonstrated an in vitro interaction between Alix and HIV-1 Gag (18, 19). Additionally, one study has found a functional role for this motif with respect to virus release in the context of a truncated (“minimal”) Gag protein (18). In the current study, ITC and SPR results demonstrate for the first time that Alix 364–716 interacts with the YPXL motif in HIV-1. Additionally, the relative insensitivity of YPXL mutant release and Gag processing to expression of Alix 364–716 as compared with that for WT suggests that the HIV-1 Gag YPXL motif interacts with Alix 364–716 in vivo.

As mentioned in the Introduction, previous studies have established the modular organization of Alix by clearly identifying two func-

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**TABLE 1**

| HIV-1 p6 (ITC) | EIAV p9 (SPR) |
|---------------|--------------|
| K<sub>d</sub> (μM) | K<sub>d</sub> (μM) |
|---|---|
| WT | 8.4 ± 0.4 | WT | 7.6 ± 0.3 |
| E34A | 6.5 ± 0.9 | L22A | ND<sup>a</sup> |
| L35A | 10.6 ± 2.0 | Y22A | ND<sup>a</sup> |
| Del 34, 35 | 174.8 | Y23F | 7.6 ± 0.3 |
| Y36A | ND<sup>a</sup> | P24A | ND<sup>a</sup> |
| Y36F | 10.1 | L26A | ND<sup>a</sup> |
| P37A | 166.3 | L26R | ND<sup>a</sup> |
| Y36F<sup>b</sup> | 5.9 | L29A | ND<sup>a</sup> |
| L41A | ND<sup>a</sup> | H11006 | M |
| L41R | 190.0 | 0.4 WT | 7.6 |
| L44A | ND<sup>a</sup> | 0.3 EIAV p9 | 0.1 |
| HIV-1 p6 16-mer<sup>c</sup> | 4.7 ± 0.1 | 0.3 HIV-1 p6 WT | 2.2 ± 0.1 |
| EIAV p9 15-mer<sup>d</sup> | 5.9 ± 0.7 | 0.3 EIAV p9 WT | 2.2 ± 0.1 |

*<sup>a</sup>Apparent dissociation constant calculated from fitting the data to a one-site model.

*<sup>b</sup>ND, either binding was not detectable, or the K<sub>d</sub> was not determined because binding was too weak to obtain reliable quantification.

<sup>c</sup>HIV-1 p6 16-mer (DYKELPDSLRSGLFN).

<sup>d</sup>EIAV p9 15-mer (JYFDLXYLYPDEISEI).

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**FIGURE 7.** ITC of WT and mutant HIV-1 p6 and WT EIAV p9. ITC analysis of the binding of WT and mutant HIV-1 p6 to Alix 364–716 in solution. Inset, raw heat change elicited by successive injections of WT HIV-1 p6 into Alix 364–716.

**FIGURE 8.** Modular structure and function of Alix. The crystal structure of the homologous Bro1 domain of yeast Bro1 is shown at the N terminus, followed by Alix 364–716 and the Pro-rich region, all drawn to scale. Interactions are shown with their known or putative mapping to individual domains.
The Viral Late Motif Binding Domain of Alix

tional domains: an N-terminal Bro domain that binds to CHMP3 of the ESCRT-III protein complex (37), and a C-terminal proline-rich domain that binds a number of factors, including those involved in endocytosis (38–40) and apoptosis (39, 41–43) (Fig. 8). The current study has established that the region of Alix spanning amino acids 364–716 (highlighted in blue in Fig. 8) contains the determinants responsible for binding to HIV-1 p6. These results are consistent with findings from Chen and co-workers (16), who identified a fragment of Alix spanning amino acids 409–715 that both binds EIAV p9 and inhibits EIAV production in a YPXL motif-dependent manner. This binding site in Alix is distinct from those in Tsg101 and Nedd4-like proteins that interact with P(T/S)AP and PPPY late domains, respectively (2–4, 11).

Full-length HIV-1 p6 and EIAV p9 proteins and short peptides containing their YPXL motifs bind directly to Alix 364–716 with micromolar affinities. These moderate affinities are typical of those seen in trafficking interactions. The affinities are similar to the 25 μM $K_d$ for the HIV-1 p6 P(T/S)AP-containing peptide binding to its target, the ubiquitin E2 variant domain of Tsg101 (11). Essentially all of the binding affinity is encoded within the short peptide motifs, since very little difference was seen between the affinities of full-length p6 and p9 versus the short synthetic peptides for Alix 364–716.

A comparative approach was taken to analyze specificity determinants for Alix 364–716 binding. The key residues in the HIV-1 and EIAV motifs are nearly identical and can be summarized as (L)[FY]Px$_{4–3}$LXX[IL]. We number these positions taking the anchoring aromatic residue as 0 and excluding the two additional spacer residues present only in the HIV-1 sequence. The only significant difference is that the Leu$^{-1}$ in the motif, shown in parentheses, appears to be more important for EIAV Gag than for HIV-1 Gag binding. Our observations are consistent with earlier work that showed that Leu$^{-1}$, Tyr$^{0}$, Pro$^{1}$, and Leu$^{3}$ of EIAV and Pro$^{1}$, Leu$^{3}$, and Leu$^{6}$ of HIV-1 are qualitatively required for interaction with full-length Alix and for viral release (18). The common requirements for binding suggest a single structural binding mode shared by HIV-1 and EIAV. It seems likely that human proteins with similar motifs should exist that interact with Alix 364–716, although no clear example has been reported to date.

An intriguing aspect of the data presented herein is that the impact of Alix 364–716 on WT HIV-1 release is strikingly different from that of mutations in p6 that disrupt Alix binding. Specifically, WT virus undergoes severely altered processing of Pr55Gag to p25/p24 and diminished virus release upon Alix 364–716 expression, whereas mutation of the Alix binding site in the absence of Alix 364–716 expression leads to accumulation of Gag processing intermediates p41 and p25 but no diminution in virus release. Furthermore, Alix 364–716 expression induced a virion morphology that is quite distinct from that observed for the Alix binding site mutants L41A and L41R in the absence of Alix 364–716. Taken together, these data suggest that the phenotypes induced by Alix 364–716 are not simply due to inhibition of Gag/Alix binding but rather could result from a more global disruption of Gag assembly, processing and budding activities. The reduced levels of cell-associated WT Gag observed in the presence of Alix 364–716 suggest that in addition to the budding block imposed by the Alix fragment, its binding to Gag might also result in Gag destabilization or degradation. Thus, although these data do not indicate the precise role of Alix in HIV-1 replication, this study does identify Alix 364–716 as a potent, Alix binding site-specific inhibitor of HIV-1 assembly and release. Alix 364–716 thus provides a new tool for understanding the basic biology of Gag processing and virus release.

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