

Structural and Biochemical Studies of ALIX/AIP1 and Its Role in Retrovirus Budding

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SUMMARY

ALIX/AIP1 functions in enveloped virus budding, endosomal protein sorting, and many other cellular processes. Retroviruses, including HIV-1, SIV, and EIAV, bind and recruit ALIX through YPX_nL late-domain motifs (X = any residue; n = 1–3). Crystal structures reveal that human ALIX is composed of an N-terminal Bro1 domain and a central domain that is composed of two extended three-helix bundles that form elongated arms that fold back into a “V.” The structures also reveal conformational flexibility in the arms that suggests that the V domain may act as a flexible hinge in response to ligand binding. YPX_nL late domains bind in a conserved hydrophobic pocket on the second arm near the apex of the V, whereas CHMP4/ESCRT-III proteins bind a conserved hydrophobic patch on the Bro1 domain, and both interactions are required for virus budding. ALIX therefore serves as a flexible, extended scaffold that connects retroviral Gag proteins to ESCRT-III and other cellular-budding machinery.

INTRODUCTION

Many enveloped RNA viruses use short peptide motifs, termed “late domains,” to recruit cellular factors that facilitate budding (reviewed in Bieniasz, 2006; Demirov and Freed, 2004; Morita and Sundquist, 2004). Two of the best characterized late domains are the PTAP motif, which binds and recruits TSG101 (tumor susceptibility gene 101; Demirov et al., 2002; Garrus et al., 2001; Gottlinger et al., 1991; Huang et al., 1995; Martin-Serrano et al., 2001; VerPlank et al., 2001), and the YPX_nL motif (where X can vary in sequence and length), which binds ALIX/AIP1 (ALG-2-interacting protein X; Chen et al., 2005; Puffer et al., 1997; Strack et al., 2003; Vincent et al., 2003). YPX_nL

late domains can vary in sequence and can function alone or together with PTAP late domains. For example, the structural p6^{Gag} protein of HIV-1_{NL4-3} contains ₇PTAP₁₀ and ₃₆YPLASL₄₁ late domains that function in tandem, whereas the analogous EIAV p9^{Gag} protein contains a single ₂₃YPDL₂₆ late domain. In principle, multiple late domains could synergistically enhance virus release and/or expand viral tropism.

Both TSG101 (Vps23p in yeast) and ALIX (Bro1p) help sort membrane proteins into vesicles that bud into the lumen of multivesicular bodies (MVB), which supports the idea that virus budding and MVB vesicle formation are highly related processes. The requirements for MVB protein sorting and vesicle formation are best understood in yeast, where the process requires the action of at least 18 different “Class E” proteins. Most, but not all, Class E proteins are stable subunits of the three ESCRT complexes (endosomal sorting complexes required for transport; Hurley and Emr, 2006). Humans have at least one homolog of every yeast Class E protein, and MVB vesicle sorting therefore appears to be a highly conserved process, albeit one that occurs with considerably greater complexity in mammals.

TSG101 functions as the central subunit of ESCRT-I, which recognizes ubiquitylated membrane-protein cargoes and helps recruit the downstream machinery necessary for protein sorting and MVB vesicle formation (Hurley and Emr, 2006). This downstream machinery includes the ESCRT-III and VPS4-LIP5 complexes, which appear to be intimately involved in the actual mechanics of protein sorting and vesicle formation. ALIX is also a Class E protein and can interact directly with both ESCRT-I and ESCRT-III but is not a stable subunit of either complex (reviewed in Odorizzi, 2006). The yeast homolog Bro1p also recruits the deubiquitylating enzyme Doa4p, which removes ubiquitin from cargoes as they are sorted into MVB vesicles (Dejournett et al., 2006; Luhtala and Odorizzi, 2004).

In addition to its roles in virus budding and MVB cargo sorting, ALIX has also been implicated in a number of other important cellular processes, including (1) MVB vesicle fission and back fusion via regulation of the conical lipid,

lysobisphosphatidic acid (LBPA; reviewed in van der Goot and Gruenberg, 2006); (2) endocytosis via interactions with the membrane-curvature-sensing endophilins (Chattellard-Causse et al., 2002; Gallop and McMahon, 2005); (3) cell-surface-receptor downregulation via direct interactions with cell-surface receptors (Geminard et al., 2004) and through antagonism of the Cbl-SETA/CIN85-endophilin complex (Schmidt et al., 2004); (4) spatial distribution of endosomes via regulation of cortical actin (Cabezas et al., 2005); (5) cell motility/adhesion via interactions with FAK (focal adhesion kinase), PYK2 (proline-rich tyrosine kinase 2; Schmidt et al., 2003), and possibly also RabGAPLP (*Rab* GTPase-activating protein-like protein; Ichioka et al., 2005); (6) apoptosis via interactions with the calcium-binding EF-hand protein ALG-2 (apoptosis-linked gene-2; reviewed in Sadoul, 2006); (7) regulation of the JNK signaling pathway via interactions with ALG-2 and the ubiquitin E3 ligase POSH (plenty of SH3 domains; Tsuda et al., 2006). The latter observation is of particular interest because POSH is also required for HIV-1 release (Alroy et al., 2005), which raises the possibility that the ALIX-POSH-ALG-2 complex could function in HIV-1 budding.

In summary, it is now apparent that ALIX performs a series of important functions in the endosomal pathway, in cytoskeletal dynamics, and in enveloped virus budding. However, the mechanistic bases for these seemingly diverse ALIX functions are not known. Our studies were therefore undertaken with the goals of elucidating the structure of ALIX, determining how it interacts with retroviral Gag proteins, and testing its requirements for functioning in virus budding.

RESULTS

Structural Studies of Human ALIX

As illustrated in Figure 1A, human ALIX can be subdivided into three regions; the Bro1 domain (residues 1–358), the “V” domain (362–702), and the proline-rich region (PRR; 703–868). Constructs containing the PRR did not express well in *E. coli*, but constructs spanning the Bro1 and V domains could be expressed and purified. A series of ALIX_{Bro1-V} proteins with loop mutations were surveyed to identify a protein that would crystallize. The KK_{268,269}YY mutation enabled crystallization (apparently because Tyr268 makes an important crystal contact), and the structure of this protein (hereafter termed ALIX_{Bro1-V}) was determined at a resolution of 3.3 Å ($R_{\text{free}} = 31.7\%$; Figures 1B and S1; Table S1). Isolated wild-type Bro1 and V domains were also crystallized and their structures were determined at higher resolutions (2.55 Å, $R_{\text{free}} = 27.4\%$ and 2.6 Å, $R_{\text{free}} = 30.2\%$, respectively; see Figures 1C–1E). The V-domain crystals contained two molecules in the asymmetric unit, and the different structures therefore provided three independent views of the ALIX V domain.

Global Architecture of ALIX_{Bro1-V}

As illustrated in Figure 1B, the ALIX_{Bro1-V} protein adopts an extended conformation in which the Bro1 and V domains

form discrete elements. Like its yeast analog, Bro1p (Kim et al., 2005), ALIX_{Bro1} is shaped like a banana, with a long dimension of ~ 100 Å. The domain is organized about a core tetratricopeptide repeat (TPR) composed of three helical hairpins that associate into a right-handed superhelix (Figure 1C). The ALIX V domain is composed of two extended arms that fold back on themselves at an angle of $\sim 30^\circ$ to form a V. The arms are 77 Å (arm1) and 90 Å (arm2) in length, and each is organized about an extended three-helix bundle (Figures 1D and 1E). The V-shaped conformation brings the N-terminal Bro1 domain and the C-terminal PRR (not present in the structure) into spatial proximity, thereby explaining how Src kinase can simultaneously dock to the Bro1 domain and phosphorylate Tyr residues in the ALIX C-terminal tail (Schmidt et al., 2005).

ALIX_{Bro1-V} has a highly asymmetric shape because the long axes of the Bro1 and arm1 elements connect in parallel. As a result, ALIX_{Bro1-V} is ~ 150 Å in its longest dimension but less than 50 Å in its other two. Small-angle X-ray scattering profiles fit the crystal model well (R.D.F. and H. Tsuruta, unpublished data), which indicates that the crystal structure provides a good model for the solution structure of the uncomplexed protein. Nevertheless, we speculate that the different domains could change their relative orientations in response to environmental cues like ligand binding because the Bro1 and V domains are connected by a single tripeptide linker (₃₅₉VPV₃₆₁) that makes only limited noncovalent contacts (Figures 1B and 2A). Moreover, the V-domain arm trajectories vary in the three different structures, which results in relative displacements of up to 10 Å at their tips (Figure 2B). Finally, the loop region between the two arms of the V domain also has the potential to act as a hinge (Figure 2C and Discussion). Thus, the overall impression is that ALIX_{Bro1-V} is a scaffold composed of extended domains that may reorient in response to ligand binding.

Structure of the Bro1 Domain

The three helical hairpins that comprise the ALIX_{Bro1} TPR core ($\alpha 4/5$, $\alpha 6/7$, and $\alpha 8/9$) are each ~ 50 residues in length. They form a right-handed solenoid with a rotation of $\sim 20^\circ$ between each helical pair. The core is flanked on one side by $\alpha 10$ (left in Figure 1C) and on the other by a small β sheet ($\beta 1$, $\beta 2$) and a three-helix bundle ($\alpha 1-3$). Both the N and C termini adopt extended conformations that traverse opposite sides of the domain, with the first 17 residues extending along the convex surface and the final 43 residues extending across the concave surface.

As expected, human ALIX_{Bro1} generally resembles its yeast counterpart (Kim et al., 2005), with an overall rmsd of 2.8 Å over 338 equivalent C α positions (Figure S2). The character of the domain surface is also largely conserved, with two exposed hydrophobic patches centered about Tyr319 and Phe199. The first patch forms the docking site for Src kinase when Tyr319 is phosphorylated (Schmidt et al., 2005), and the second patch forms the binding site for the CHMP4B subunits of the ESCRT-III complex (Kim et al., 2005 and see below). A strongly

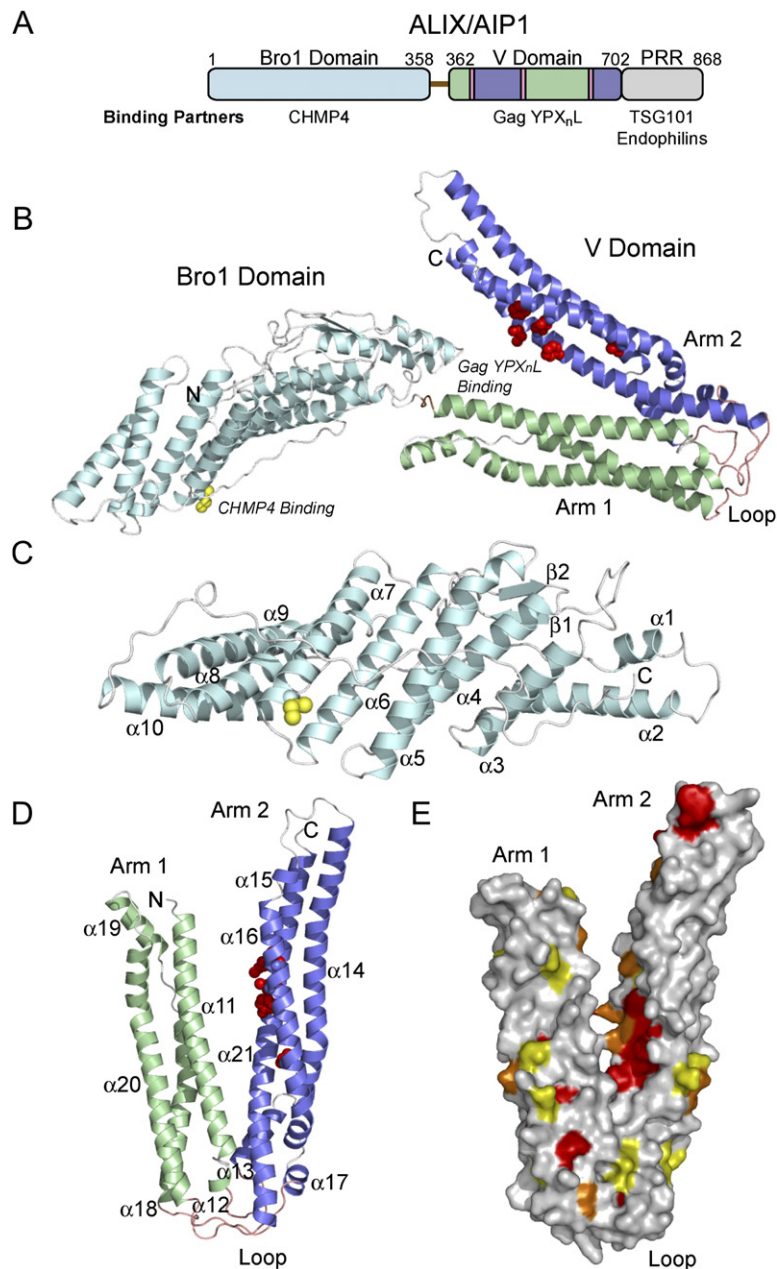


Figure 1. Structure of ALIX_{Bro1-V}

(A) shows domain structure of human ALIX. Sequences that compose the different elements are color coded here (and throughout) as follows: Bro1, turquoise; Bro1-V linker, brown; V-domain arm1, green; V-domain loop, salmon; V-domain arm2, blue; and Proline-rich region (PRR), gray.

(B) Ribbon representation of ALIX_{Bro1-V} is shown. Residues implicated in CHMP4 binding (Ile212; yellow) or YPX_nL binding (Phe495, Val498, Ala509, Phe676, Leu680, and Ile683; red) are highlighted. The Bro1 and V domains are connected by a short hydrophobic linker (₃₅₉VPV₃₆₁). The limited set of interdomain hydrophobic packing interactions is made by Phe24 (α 1, Bro1), Val359, Pro360, Val361, Val363 (α 1, arm1, and V), and Leu585 (α 19, arm1, and V).

(C) This ribbon diagram of ALIX_{Bro1} shows the secondary-structure-labeling scheme. The view is from the underside of the orientation shown in (A).

(D) shows a ribbon diagram of ALIX_V showing the secondary-structure-labeling scheme. As compared to (A), this orientation is rotated clockwise by $\sim 60^\circ$ relative to a line perpendicular to the plane of the paper.

(E) This surface rendering shows ALIX_V sequence conservation. ALIX_V sequences from seven divergent species were aligned using ESPript (Gouet et al., 1999); residues with scaled similarity scores are color coded as follows: 85–100 are red, 68–84 are orange, and 50–67 are yellow.

electropositive patch at one end of the yeast Bro1 domain is also basic in the human protein, albeit to a lesser extent.

The human and yeast Bro1 domains differ most at one end of the domain (right in Figure 1C), where N- and C-terminal elements differ in secondary structure and packing (Figure S2). Specifically, the C terminus of yeast Bro1p forms three consecutive helical segments that cross the concave side and then turn up into the domain, whereas the equivalent residues in the human protein (342–358) form an extended strand that traverses a similar path but terminates earlier. Thus, the C-terminal helix of the yeast protein packs between α 3 and the α 1–2 hairpin (ALIX_{Bro1} numbering), whereas the human Bro1 C terminus is

shifted by ~ 10 Å, and the first three helices of human ALIX_{Bro1} collapse into a three-helix bundle (a maximal relative shift of ~ 12 Å). It appears possible that these distinct C-terminal conformations could alter the relative orientations of the Bro1 and V domains in the different proteins.

The Structure of the ALIX V Domain

Both arms of the ALIX V domain are composed of extended three-helix bundles, although a series of short breaks subdivide the major helices into 11 different segments (Figures 1D, S1, and S3). The topology of the V domain is notable in that the polypeptide chain crosses the arm1/arm2 “loop” region three times in the course of

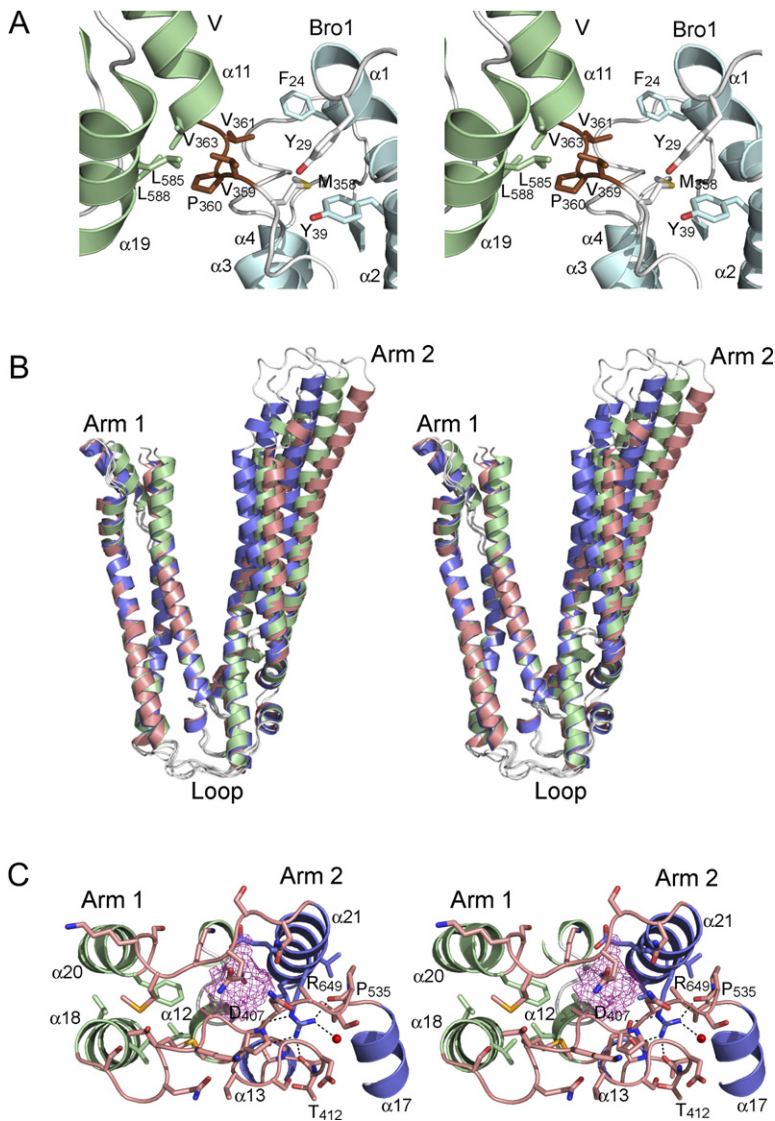


Figure 2. Interdomain Linkers and Conformational Flexibility of ALIX_{Bro1-V}

(A) Stereoview showing the linker between the Bro1 and V domains is shown. Secondary structures are color coded as in Figure 1B, and residues that contact the ₃₅₉VPV₃₆₁ linker are shown explicitly.

(B) This stereoview illustrates conformational variability in different V-domain structures. The different trajectories of arm2 were visualized by superimposing only the arm1 regions from crystals of ALIX_{Bro1-V} (salmon) and from the two different molecules in the ALIX_V asymmetric unit (green and blue). The only other significant difference between the structures was in the position of Trp476 residue (α 4/5 loop), which flips out of the core of arm2 in one of the two ALIX_V structures to make a crystal contact.

(C) shows a stereoview illustrating the three-stranded loop region that connects the two arms of the V domain. Secondary structures are color coded as in Figure 1B. Side chains within the loop and adjacent residues are shown, and the stabilizing interactions of the Arg649 residue are shown explicitly. The purple cage denotes a pocket that appears to be occupied by a mix of ordered and disordered water molecules. The view is from the bottom of (B).

building the two helical bundles (Figure S3). Hence, the two arms are highly interconnected in primary sequence, and the entire V domain likely represents a single functional entity.

The two V-domain arms are primarily stabilized by hydrophobic side-chain-packing interactions that are based upon heptad repeats and that adopt canonical “knobs into holes” side-chain packing. This explains why the domain scored highly in coiled-coil prediction algorithms (Kato et al., 2003; Odorizzi et al., 2003). In contrast, the three strands in the loops connecting arm1 and arm2 are stabilized almost exclusively by hydrophilic interactions, most of which are mediated by backbone atoms. An illustrative example is provided by the highly conserved Arg649 residue (α 21, arm2), which forms a series of interactions that buttress the underside of the loop region; these include a buried salt bridge with Asp407 (α 12/13 loop) and hydrogen-bonding interactions with a buried

water molecule and with the backbone carbonyl oxygen atoms of Pro535 (α 17/18 loop) and Thr412 (α 12/13 loop). The loop region also encloses a hydrophilic interior cavity of $\sim 67 \text{ \AA}^3$ (purple in Figure 2C).

The V domain exhibits intrinsic conformational flexibility, as revealed by comparisons of the relative positions of the two arms in the different crystal structures. Isolated arm1 structures from the crystallographically independent models overlap well (rmsd = 1.4 \AA), and the same is true for the arm2 structures, but overlapping on either arm causes the other to “fan out” into different positions. This is illustrated in Figure 2B for the case of overlap on arm1, where the relative arm2 displacements reach up to 10 \AA at their distal ends. These global differences in the structures arise from small, cumulative changes in the interhelical packing interactions along both arms rather than from a dramatic reorganization of the loop region or elsewhere. Thus, the structures demonstrate that the V-domain arm

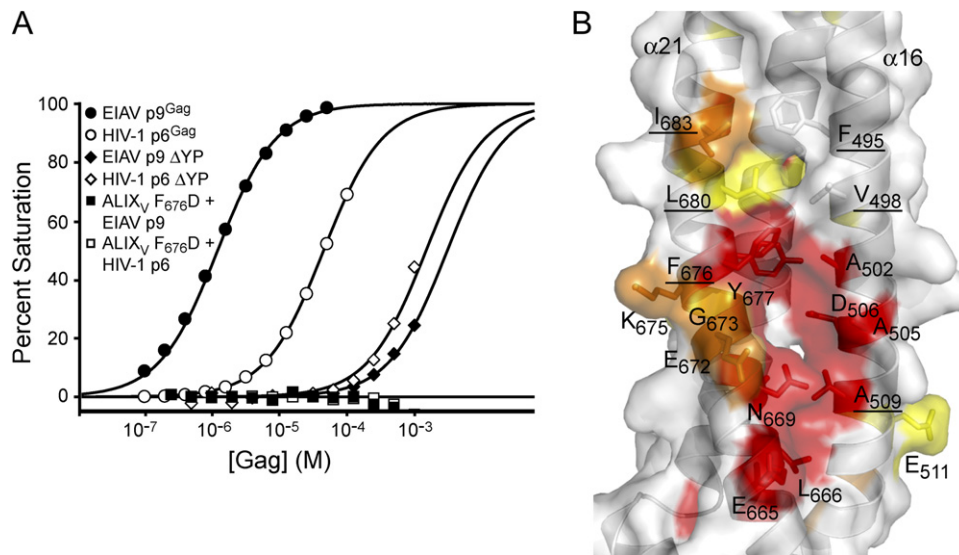


Figure 3. Identification of the YPX_nL-Binding Site on ALIX_V

(A) Biosensor binding isotherms for purified HIV-1 p6^{Gag} and EIAV p9^{Gag} constructs binding to immobilized ALIX_V domain constructs are shown. (B) YPX_nL-binding site on ALIX_V arm2 is shown here as viewed from arm1. Residue conservation is color coded as in Figure 1E. Underlined residues were mutated and tested for YPX_nL-binding activity and/or for activity in the HIV-1 ΔPTAP rescue assay (see text).

positions can vary, although the biological significance of this observation remains to be elucidated.

The ALIX V Domain Binds HIV-1 p6^{Gag} and EIAV p9^{Gag} YPX_nL Late Domains

ALIX binds and facilitates budding of retroviral Gag proteins that contain YPX_nL late domains, but the interaction site is not yet known. We therefore performed biosensor binding experiments to map the YPX_nL-binding site and measure the binding affinities of the HIV-1 p6^{Gag} and EIAV p9^{Gag} proteins. As shown in Figure 3A, the immobilized ALIX V domain bound both HIV-1 p6^{Gag} and EIAV p9^{Gag}, with dissociation constants of $59 \pm 15 \mu\text{M}$ and $1.2 \pm 0.3 \mu\text{M}$, respectively. In both cases, binding was specific for the YPX_nL late domain because YP to SR mutations (termed ΔYP; Strack et al., 2003) reduced binding affinities substantially (>15-fold). Very similar binding data were also obtained for the ALIX_{Bro1-V} construct, which indicates that the Bro1 domain did not contribute to the binding interaction (Table S2).

Close inspection of the V-domain surface revealed a highly conserved hydrophobic groove on arm2 between helices 16 and 21, centered about Phe676, that was a strong candidate for the YPX_nL late-domain-binding site (Figures 1D, 1E, 3B, and S4). This site is exposed to solvent but is located near the base of the V, where the two arms are separated by only 5–10 Å. Phe676 sits at one end of a deep pocket (~10 Å) that is lined by a series of highly conserved residues (highlighted in red in Figure 3B). A shallower hydrophobic groove also extends ~7 Å above Phe676, although the residues that line this

half of the site are less well conserved (Figures 3B, S1, and S4).

Mutational analyses confirmed that a series of conserved residues within the putative YPX_nL-binding site on arm2 were required for full-affinity HIV-1 p6^{Gag} and EIAV p9^{Gag} binding. As shown in Figure 3A, the Phe676Asp mutation abolished any detectable binding to either HIV-1 p6^{Gag} or EIAV p9^{Gag}, which represents a >1000-fold reduction in EIAV p9^{Gag} binding affinity. Mutations in two other binding-groove residues (Val498Asp and Ile683Asp) also reduced HIV-1 p6^{Gag} and EIAV p9^{Gag} binding affinities substantially (≥ 20 fold; see Table S2). These data indicate that retroviral Gag YPX_nL late domains bind the V domain of ALIX at a conserved site that spans the side and interior face of arm2.

Requirements for ALIX in EIAV and HIV-1 Release

Previous studies have indicated that ALIX can function in the release of both EIAV and HIV-1 (Chen et al., 2005; Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). Importantly, however, EIAV p9^{Gag} contains a single known late domain (the ₂₃YPDL₂₆-ALIX site), whereas HIV-1 p6^{Gag} contains two late domains: a ₇PTAP₁₀-TSG101 site and a ₃₆YPLASL₄₁-ALIX site. We therefore assessed the relative importance of ALIX for the release of both EIAV and HIV-1. As shown in Figure 4A, the release of virion-associated Gag-derived CA protein from HeLa cells expressing a wild-type EIAV vector was readily detected in western blot assays (lane 1, upper panel), but the ΔYP mutation in the p9^{Gag} ALIX-binding site reduced EIAV particle release 12-fold (compare lanes 1 and 2, panel 1). ALIX depletion similarly reduced the

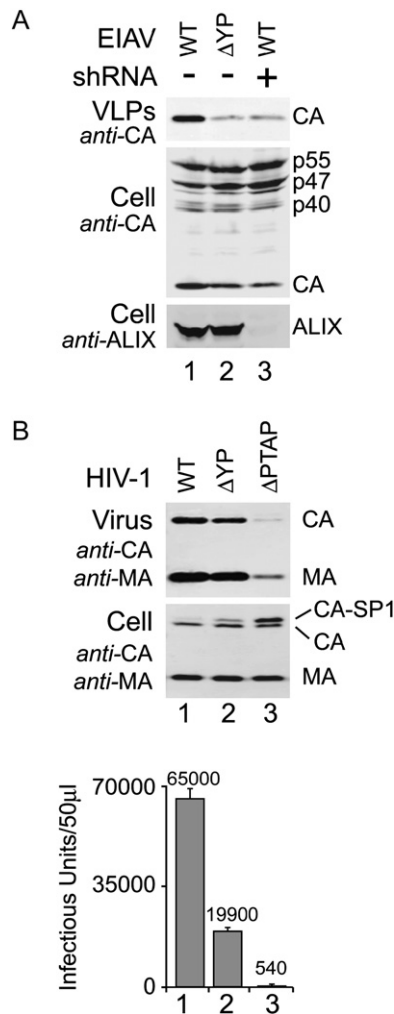


Figure 4. Requirement for ALIX in EIAV and HIV-1 Budding

(A) ALIX requirements for EIAV vector budding are shown as follows: lanes 1, wild-type (WT) EIAV vector expressed in wild-type HeLa cells (positive control); lanes 2, EIAV vector encoding a $_{23}SR_{24}$ mutation in the $_{23}YPDL_{26}$ late domain of $p9^{Gag}$ (ΔYP) expressed in wild-type HeLa cells; and lanes 3, wild-type EIAV vector expressed in cells depleted of ALIX using shRNA. Western blots are shown as follows: panel 1, virus-like particle (VLP) production (anti-CA antibody) reports the amount of successful budding and panel 2, cellular Gag protein levels (anti-CA antibody) control for expression. Note that CA corresponds to the processed central domain of Gag, p55 corresponds to full-length Gag protein, and p40 and p47 are intermediate Gag cleavage products. Cellular Gag levels were similar in all cases, although the processing was somewhat delayed in the ΔYP mutant and in the absence of ALIX. Panel 3 shows cellular ALIX levels (anti-ALIX antibody).

(B) The relative importance of ALIX and TSG101 binding for HIV-1 virus budding is shown. Lanes 1 show wild-type (WT) HIV-1 expressed in 293T cells (positive control), lanes 2 show HIV-1 encoding a $_{36}SR_{37}$ mutation in the $_{36}YPLASL_{41}$ late domain of $p6^{Gag}$ (ΔYP), and lanes 3 show wild-type HIV-1 encoding a $_{7}LIRL_{10}$ mutation in the $_{7}PTAP_{10}$ late domain of $p6^{Gag}$ ($\Delta PTAP$). Western blots show virus production (panel 1) and cellular Gag protein levels (panel 2, anti-CA and anti-MA antibodies; MA is the processed N-terminal domain of Gag). Panel 3 shows viral titers measured in a single-cycle MAGIC assay (errors are standard deviations from three separate infectivity experiments). Note

release of wild-type EIAV 10-fold (compare lanes 1 and 3, upper panel). shRNA depletion of ALIX from HeLa cells was very efficient (lane 3, bottom panel), and EIAV Gag expression was not significantly affected by either the $p9^{Gag}$ ΔYP mutation or by ALIX depletion (middle panels). Gag processing was inhibited slightly, however, which is reminiscent of the Gag-processing delay observed upon inhibition of HIV-1 budding (Gottlinger et al., 1991). We therefore conclude that the $_{23}YPDL_{26}$ -ALIX interaction plays a critical role in enhancing EIAV Gag release, which is in good agreement with previous studies (Martin-Serrano et al., 2003; Puffer et al., 1997; Strack et al., 2003).

The importance of ALIX for HIV-1 release was also tested by measuring the effect of the ΔYP mutation in the ALIX-binding site of HIV-1 $p6^{Gag}$. As shown in Figure 4B, this mutation reduced HIV-1 Gag release from 293T cells, as measured in both a western blot assay (compare lanes 1 and 2, panel 1) and by viral titers in a single-cycle MAGIC infectivity assay (lanes 1 and 2, panel 3). However, the reductions in release and infectivity were modest (~ 3 -fold). In comparison, a mutation that blocked the $_{7}PTAP_{10}$ -TSG101 interaction ($PTAP_{7-10}LIRL$, termed $\Delta PTAP$) had a much more profound effect on HIV-1 release and reduced infectious titers more than 100-fold (Figure 4B, compare lanes 1 and 3). Neither late-domain mutation affected Gag protein expression, although both again delayed Gag processing, as evidenced by intracellular accumulation of the CA-SP1-processing intermediate, with the more profound effect again seen for the $\Delta PTAP$ mutation (Figure 4B, central panel). These experiments indicate that ALIX and TSG101 both enhance the release of wild-type HIV-1 from 293T cells but that the virus depends much more heavily upon the $_{7}PTAP_{10}$ -TSG101 interaction. These studies again generally agree well with previous mutational analyses that utilized different HIV-1 constructs and cell types and were performed before the discovery of the $p6^{Gag}$ $_{36}YPLASL_{41}$ -ALIX interaction (Demirov et al., 2002; Gottlinger et al., 1991; Huang et al., 1995).

ALIX Overexpression Rescues Release and Infectivity of HIV-1 $\Delta PTAP$

The experiments described above demonstrate that the $p9^{Gag}$ $_{23}YPDL_{26}$ -ALIX late-domain interaction plays a major role in facilitating EIAV release, yet the $p6^{Gag}$ $_{36}YPLASL_{41}$ -ALIX interaction does not efficiently substitute for the $_{7}PTAP_{10}$ -TSG101 interaction in supporting HIV-1 release. We hypothesized that this apparent discrepancy might reflect a reduced ability of HIV-1 to recruit ALIX owing to the lower affinity of the $p6^{Gag}$ YPX_nL -binding site. We therefore tested whether the inefficient release of the HIV-1 $\Delta PTAP$ virus from 293T cells could be "rescued" by increasing intracellular ALIX concentrations.

that cellular CA and MA levels were similar in all cases but that the CA-SP1-processing intermediate accumulated in the ΔYP and $\Delta PTAP$ constructs, which is a typical diagnostic of virus-budding inhibition.

As shown in Figure 5, HIV-1 Δ PTAP release and infectivity were indeed stimulated very dramatically (\sim 25-fold) by ALIX overexpression (Figure 5, panels 1 and 4, compare lanes 1 and 2). Hence, raising cellular ALIX levels could restore HIV-1 Δ PTAP infectivity to within \sim 20% of the wild-type virus. Importantly, ALIX overexpression did not alter intracellular Gag expression or processing (panel 2), which demonstrates that the enhancement occurred at late stages in particle assembly or budding. Control experiments also confirmed that the p6^{Gag}₃₆YPLASL₄₁-ALIX interaction was responsible for this enhancement because ALIX overexpression did not stimulate the release of an HIV-1 Δ PTAP virus that also lacked a functional ALIX-binding site (compare lanes 2 and 4). Indeed, the secondary Δ YP mutation actually reduced HIV-1 Δ PTAP release and infectivity by an additional 5-fold (compare lanes 3 and 4 to lane 1) presumably because the mutation also inhibited the ability of *endogenous* ALIX to mediate low-level release. These experiments demonstrate that ALIX can replace TSG101 in its role of supporting the efficient release of infectious HIV-1, provided ALIX is present at sufficient levels to overcome the modest affinity of the ₃₆YPLASL₄₁-ALIX interaction.

ALIX YPX_nL-Binding Activity Is Required for Virus Release

The rescue of HIV-1 Δ PTAP release and infectivity upon ALIX overexpression provided a convenient assay for testing which ALIX activities were required for functional virus release. This assay was initially used to test whether ALIX mutations that inhibited YPX_nL binding *in vitro* also inhibited virus release through the ALIX pathway. As described previously, overexpression of wild-type ALIX enhanced the release and infectivity of HIV-1 Δ PTAP \sim 25-fold (Figure 6A, compare lanes 1 and 2). In contrast, ALIX proteins with point mutations that inhibited YPX_nL binding (Val498Asp, Phe676Asp, and Ile683Asp) failed to rescue HIV-1 Δ PTAP release or infectivity (compare lanes 2 and 3–5). These mutations did not affect ALIX expression or stability (panel 3), nor did they affect cellular Gag levels (panel 2). Similar data were obtained for a series of other mutations within the YPX_nL-binding site (Phe495Asp, Val509Asp, and Leu680Asp), whereas mutations in a second conserved hydrophobic patch located on arm1 (Leu401Asp, Ile405Asp, and Leu556Asp) did not diminish ALIX rescue significantly ($<$ 2-fold, data not shown). These data reinforce the idea that ALIX binds YPX_nL late domains within the site shown in Figure 2B and demonstrate that ALIX mutations that impair YPX_nL binding also inhibit virus release and infectivity.

ALIX Recruitment of CHMP4/ESCRT-III Is Required for HIV-1 Δ PTAP Release

In addition to binding YPX_nL late domains, ALIX also binds CHMP4/ESCRT-III proteins through the Bro1 domain, suggesting that ALIX may serve to connect retroviral Gag proteins to the ESCRT-III machinery (Katoh et al., 2003; Kim et al., 2005; Martin-Serrano et al., 2003; Odor-

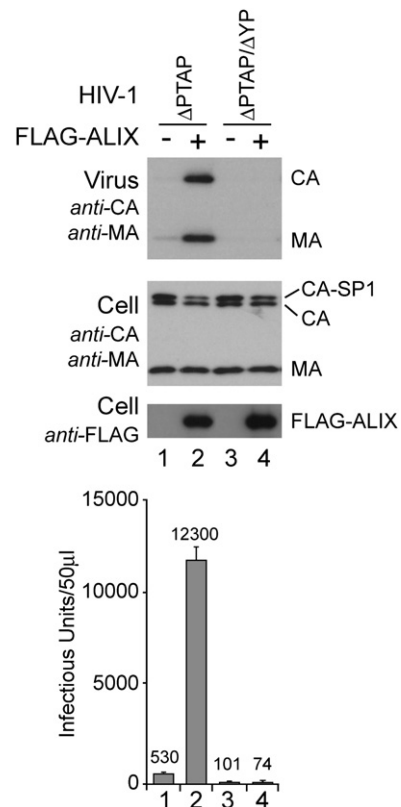


Figure 5. ALIX Overexpression Rescues HIV-1 Δ PTAP Release and Infectivity

Virus release, cellular Gag protein levels, exogenous ALIX expression, and viral infectivity are shown as analyzed by western blotting (panels 1–3) or MAGIC infectivity assays (panel 4). Lanes 1 show HIV-1 Δ PTAP cotransfected with an empty pCI-neo vector control, lanes 2 show HIV-1 Δ PTAP cotransfected with a vector expressing FLAG-ALIX, lanes 3 show HIV-1 Δ PTAP Δ YP cotransfected with an empty vector control, and lanes 4 show HIV-1 Δ PTAP Δ YP cotransfected with a vector expressing FLAG-ALIX. For reference, wild-type HIV-1 titers were typically \sim 65,000 IU/50 μ l, and overexpression raised ALIX levels \sim 50-fold over endogenous protein levels as estimated by western blotting with anti-ALIX antibodies (not shown). Error bars in infectivity assays represent standard deviations of three separate infectivity experiments.

izzi et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). We therefore tested whether the interaction between ALIX and CHMP4A was required for virus budding. Previous work showed that mutations in an exposed hydrophobic patch on yeast Bro1p inhibited Snf7p (CHMP4) binding and prevented proper MVB sorting (Kim et al., 2005). We found that a mutation within the equivalent patch on human ALIX, Ile212Asp, similarly inhibited the ALIX_{Bro1}-CHMP4A interaction, as assayed in a GST pulldown experiment (Figure 6B). As expected, wild-type ALIX_{Bro1} bound to a GST-CHMP4A fusion protein but not to GST alone (compare lanes 2 and 3). In contrast, the ALIX_{Bro1,I212D} mutant did not bind GST-CHMP4A detectably (compare lanes 3 and 6).

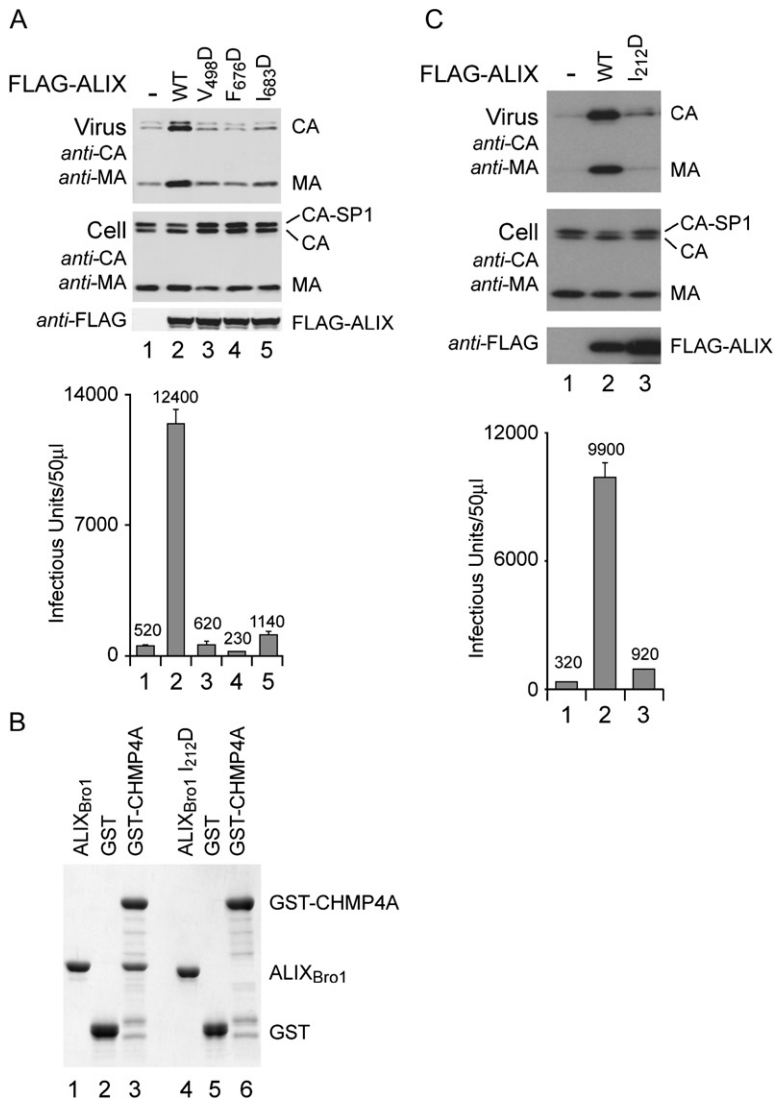


Figure 6. ALIX Mutants Lacking YPX_nL- and CHMP4-Binding Activities Do Not Support HIV-1 ΔPTAP Release and Infectivity

(A) HIV-1 ΔPTAP release and infectivity when coexpressed with an empty vector (-; lanes 1), with vectors expressing wild-type ALIX (lanes 2), or with ALIX mutants defective in YPX_nL binding (lanes 3–5) are shown. ALIX and Gag protein levels were analyzed by western blotting (panels 1–3), and infectious titers were analyzed using MAGIC assays (panel 4). (B) This GST pull-down assay shows that the ALIX_{Bro1} mutation Ile212Asp inhibits GST-CHMP4A binding. Binding experiments were performed with wild-type ALIX_{Bro1} (lanes 1–3) and the I₂₁₂D mutant (lanes 4–6). Lanes 1 and 4 show pure ALIX_{Bro1} proteins (reference markers), lanes 2 and 4 show ALIX_{Bro1} proteins binding to GST alone (negative controls), and lanes 3 and 6 show ALIX_{Bro1} proteins binding to GST-CHMP4A. (C) HIV-1 ΔPTAP release and infectivity are shown when coexpressed with an empty expression vector (-, lanes 1), with vectors expressing wild-type ALIX (lanes 2), or with the ALIX Ile212Asp mutant (lanes 3), which is defective in CHMP4A binding. In (A) and (C), HIV-1 ΔPTAP release, cellular Gag protein levels, exogenous ALIX expression, and viral infectivity were analyzed as described in Figure 4, with error bars representing standard deviations from three separate infectivity experiments.

We next tested whether the Ile212Asp mutation affected the ability of ALIX to rescue HIV-1 ΔPTAP infectivity. As shown in Figure 6C, the ALIX_{I212D} mutant expressed well (panel 3, compare lanes 2 and 3) but failed to rescue HIV-1 ΔPTAP release or infectivity significantly (top and bottom panels, compare lanes 2 and 3). Hence, the Ile212Asp mutation strongly inhibited the ability of ALIX to support HIV-1 release, implying that one essential ALIX function in virus budding is to recruit CHMP4/ESCRT-III.

Other ALIX Functions in Virus Budding

The HIV-1 ΔPTAP rescue assay was also used to survey the functional importance of four other known ALIX properties: Tyr319 phosphorylation, TSG101 binding, endophilin binding, and interactions of the C-terminal PRR. ALIX mutants defective in each of these properties were generated and tested for their ability to rescue HIV-1 ΔPTAP release. In the first case, the ALIX Tyr319Phe mutation, which

blocks phosphorylation and Src kinase binding (Schmidt et al., 2005), did not impair the rescue of HIV-1 ΔPTAP release and infectivity (Figure 7, lane 3). This result indicates that Src binding and phosphorylation do not play essential (or nonredundant) roles in ALIX virus-budding activity.

ALIX can also bind directly to the N-terminal UEV domain of TSG101 via a ₇₁₇PSAP₇₂₀ motif located in the ALIX PRR (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). This interaction is of interest because it provides a potential mechanism for association of the two known late-domain-binding partners of HIV-1. As expected, mutation of the final proline in the ₇₁₇PSAP₇₂₀ motif (Pro720Leu) eliminated TSG101 UEV binding entirely (Figure S5A). However, the ALIX_{P720L} mutant fully rescued HIV-1 ΔPTAP release and infectivity (Figure 7, lane 4), which indicates that a functional TSG101-binding site was not required for ALIX to support virus budding. Indeed, we found that ALIX overexpression rescued HIV-1 ΔPTAP release even when TSG101 was

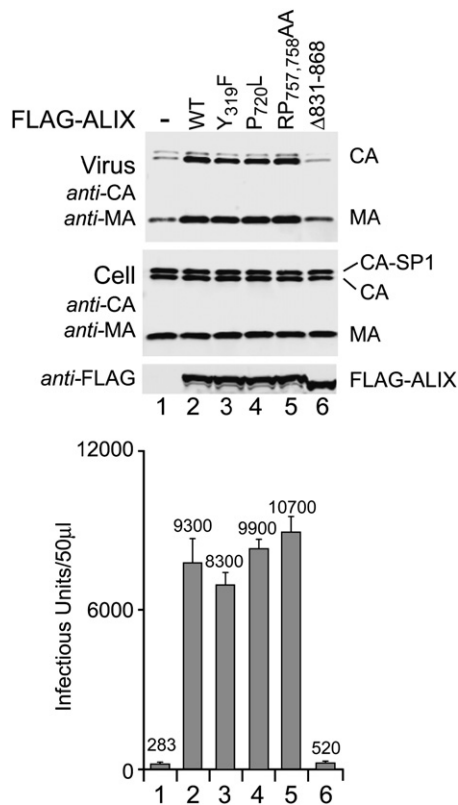


Figure 7. ALIX Requirements for HIV-1 Δ PTAP Release and Infectivity

HIV-1 Δ PTAP release and infectivity are shown when coexpressed with: an empty vector (–; lanes 1) with expression vectors for wild-type ALIX (lanes 2), with the ALIX Y₃₁₉F mutant (lanes 3), which cannot be phosphorylated on residue 319, with the ALIX P₇₂₀L mutant (lanes 4), which is defective in TSG101 binding, with the ALIX R_{P757,758AA} mutant (lanes 5), which is defective in endophilin binding, or with the ALIX Δ _{831–868} mutant (lanes 6), which lacks the final 38 residues in the PRR. HIV-1 Δ PTAP release, cellular Gag protein levels, exogenous ALIX expression, and viral infectivity were analyzed as described in Figure 4, with error bars representing standard deviations from three separate infectivity experiments.

depleted from cells (not shown), which indicates that the ALIX and TSG101 virus-budding pathways can function independently.

Endophilins represent another interesting class of ALIX-binding partners because endophilins can bind membranes and can drive (or sense) membrane curvature. Moreover, endophilin-2 binds and facilitates MLV Gag protein release (Wang et al., 2003). Endophilins bind the ⁷⁵⁵PXRPPPP₇₆₁ sequence within the ALIX PRR (Chatelard-Causse et al., 2002; Shibata et al., 2004). We employed yeast two-hybrid assays to confirm that the ALIX R_{P757,758AA} mutation inhibited binding to both endophilin-1 and -2 (Figure S5B). Despite lacking endophilin-binding activity, however, the ALIX R_{P757,758AA} mutant efficiently rescued HIV-1 Δ PTAP release and infectivity, which indicates that endophilin binding was also dispens-

able for the virus-budding function of ALIX (Figure 7, lane 5).

Finally, we tested the general importance of the ALIX PRR using several deletion mutants, the shortest of which removed just the final 38 ALIX residues (ALIX Δ _{831–868}). All of the C-terminal deletion mutants tested, including ALIX Δ _{831–868}, failed to rescue HIV Δ PTAP release and infectivity (lane 6) despite equivalent expression levels of the mutant and wild-type proteins (panel 3, compare lanes 2 and 6). We therefore conclude that ALIX PRR interaction(s) are required for late budding activity.

DISCUSSION

ALIX_{Bro1-V} Structure

The elongated Bro1 domain extends from the first arm of the central V domain, which gives ALIX_{Bro1-V} the shape of a “check mark.” This distinctive conformation explains how Src kinase can bridge the N-terminal Bro1 domain and the distal PRR tail and how it may similarly bring other Bro1- and PRR-binding proteins into close proximity. The ALIX_{Bro1-V} structure also suggests several ways in which conformational changes could function in the numerous biological roles ascribed to ALIX. Two types of domain motion are attractive possibilities: (1) variation in the relative orientation of the Bro1 and V domains, which appears possible given the limited number of contacts made by the interdomain linker (Figure 2A), and (2) variation in the trajectories of the two arms of the V domain, as implied by differences in the three different V-domain structures (Figure 2B).

In addition to the modest variation in arm orientation observed between crystal structures, we speculate that the V domain might function as a “molecular hinge,” with the structures reported here corresponding to the closed conformation. This idea is consistent with the lack of hydrophobic packing interactions throughout the loop region of the V domain (Figure 2C). The hydrophilic loop-packing interactions that do exist are well defined and geometrically constrained, however, which indicates that if the loop functions as a hinge, then activation energy will be required to break these interactions and open the V. In principle, hinge opening could be driven by posttranslational modifications and/or ligand binding and might expose new binding sites and thereby provide an elegant mechanism for transmitting signals for complex assembly and disassembly. YPX_nL late domains are obvious candidates for triggering a possible hinge-opening motion because they bind near the base of the V at a site located nearly between the two arms.

YPX_nL Binding

We have mapped the YPX_nL-binding site to a conserved, hydrophobic groove on arm2 of the ALIX V domain and demonstrated that YPX_nL binding is required for ALIX-mediated budding of both HIV-1 and EIAV. ALIX binds nearly 60× more tightly to EIAV p9^{Gag} than to HIV-1 p6^{Gag}, which is consistent with the idea that the EIAV ₂₃Y₂₃PDL₂₆ late

domain is an optimized ALIX-binding site (Vincent et al., 2003). This presumably reflects the fact that EIAV contains just a single known late domain and therefore relies heavily on ALIX to bud from cells. In contrast, the weaker HIV-1₃₆YPLASL₄₁-ALIX late-domain interaction implies that suboptimal binding must confer a selective advantage when a functional TSG101-binding site is also present. This same trend can be seen in many different SIV strains, where the absence of a TSG101-binding site appears to correlate with the presence of an optimized ALIX-binding site (and vice versa; Bibollet-Ruche et al., 2004). Hence, viral late domains are not necessarily optimized for high-affinity binding, particularly when a virus employs multiple late domains.

We also found that ALIX can efficiently support the release and infectivity of an HIV-1 construct that lacks a TSG101-binding site, provided that the cellular ALIX levels are high enough to overcome the relatively weak binding affinity. Importantly, virus release via ALIX does not require TSG101, and vice versa. Similarly, TSG101 is not required for release of a Δ p6 HIV-1 chimera that buds via a fused EIAV p9^{Gag} element (Martin-Serrano et al., 2003) nor for ALIX stimulation of murine leukemia virus budding (Segura-Morales et al., 2005). These observations all indicate that although TSG101 and ALIX can bind one another and function together in the MVB pathway, they represent independent routes out of the cell. The decisions as to which late domain(s) are employed by a particular virus, how many late domains are used, and the affinity of each binding site presumably reflect a complex optimization of the different possible exit routes from relevant host cell types.

Although the precise mechanistic role of the different cellular Class E proteins in enveloped virus budding is not yet clear, there is increasing evidence that viruses may be mimicking cellular interactions that allow membrane proteins to recruit the MVB machinery and be sorted into MVB vesicles, which ultimately leads to lysosomal degradation or release within extracellular exosomes. Interestingly, both the AP-2 adaptor complex and ALIX can bind a Tyr-based motif found in the cytoplasmic tail of the transferrin receptor (TR; although the motif does not match the YPX_nL consensus; Geminard et al., 2004). Vidal and colleagues have argued that the AP-2 interaction directs TR endocytosis, and the ALIX interaction may then direct the TR into exosomes that are released by maturing reticulocyte cells (Geminard et al., 2004). Both AP-2 and ALIX also bind the EIAV p9^{Gag} late domain and function in virus release (Chen et al., 2005), which suggests that EIAV may bud via the same pathway that sorts cellular TRs into reticulocyte-derived exosomes.

To date, the only well-documented example in which an ALIX-like protein binds a nonviral YPX_nL motif is for an ALIX homolog in *Aspergillus nidulans*, PaIA, which binds tandem YPXL/I tetrapeptide repeats within PacC, a protein involved in sensing and responding to pH changes (Vincent et al., 2003). The YPXL/I motifs are required for pH-dependent proteolytic activation of PacC, although

the precise role of PaIA in this process is not clear. Other cellular binding partners for the ALIX V domain presumably exist, but the lack of a strong consensus sequence for the YPX_nL-like binding motifs complicates their identification.

Other Functional Requirements

Our studies also show that one essential function of ALIX in virus budding is to recruit the CHMP4/ESCRT-III complex. It has been challenging to establish a direct role for the ESCRT-III proteins in virus budding because human cells express 11 distinct, but related, ESCRT-III subunits, and it is therefore difficult to differentiate between nonessential versus redundant functions (e.g., see Langelier et al., 2006). Our work provides the most direct demonstration to date that ESCRT-III recruitment is required for virus budding and is consistent with previous studies showing that dominant-negative ESCRT-III constructs can block virus release (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003) and that artificial Gag fusions that recruit ESCRT-III subunits can substitute for the₂₃YPDL₂₆ late domain in supporting EIAV p9^{Gag} release (Pineda-Molina et al., 2006). Hence, there is now increasing evidence that while enveloped RNA viruses can enter into the MVB pathway via many different binding partners, they ultimately require access to the ESCRT-III and VPS4-LIP5 complexes, which appear to be the functional machinery of MVB protein sorting/vesicle formation.

The PRR of ALIX also provides essential function(s) in virus budding. While the terminal 38 residues of ALIX could play a structural role, the high Pro and Gln content of this region (34% and 24%, respectively) makes it more likely that the polypeptide is inherently unstructured and can adopt an extended conformation that serves as the docking site for other cellular proteins. We have shown that endophilin and TSG101 binding to the PRR appear to be dispensable (or redundant) for viral budding. Determining the subset of PRR interactions that are essential for virus budding will likely shed light on the mechanistic requirements for protein sorting/vesicle formation and will further reveal how ALIX can serve as an adaptable, multidomain scaffold that links retroviral Gag proteins to essential cellular budding machinery.

EXPERIMENTAL PROCEDURES

Summary of Protein Expression, Purification, and Structure Determination

For protein-interaction studies, HIV-1 p6^{Gag} and EIAV p9^{Gag} were expressed as GST fusions in *E. coli* and purified by affinity chromatography and gel filtration chromatography (following proteolytic removal of GST). ALIX_{Bro1-V}, ALIX_{Bro1}, and SeMet ALIX_V were similarly expressed as GST or 6x-His N-terminal fusion proteins in *E. coli* and affinity purified for binding studies and crystallization. The 6x-His tag was left on crystallized ALIX_{Bro1} but removed from ALIX_{Bro1-V} and ALIX_V. The crystallized ALIX_{Bro1-V} protein had a KK_{268,269}YY mutation. Structures were determined by SAD (ALIX_V) or molecular replacement (ALIX_{Bro1} and ALIX_{Bro1-V}) and refined as summarized in Table S1. Full details of

plasmid construction, protein expression, crystallization, and structure determination are provided in the [Supplemental Data](#).

Protein-Protein Interaction Experiments

Biosensor binding experiments were performed as described (Garrus et al., 2001) with purified HIV-1 p6^{Gag}, EIAV p9^{Gag}, and HIV-1 TSG101^{UEV} proteins binding to immobilized GST-ALIX_{Bro1-V}, GST-ALIX_V, GST-ALIX_{Bro1}, and GST-ALIX_{714–723} proteins. Assay conditions and binding affinities are provided in [Table S2](#). GST pulldown experiments were performed as described (von Schwedler et al., 2003) using purified ALIX_{Bro1} proteins binding to GST or GST-CHMP4A proteins captured from clarified *E. coli* lysates.

Assays for HIV-1 ΔPTAP Release and Infectivity

293T cells (~8 × 10⁵ cells/well in 6-well plates) were transfected with 1 μg of HIV-1 ΔPTAP plasmid (Garrus et al., 2001) + 1 μg of ALIX expression vector per well (10 μl Lipofectamine 2000, Invitrogen). Cytoplasmic proteins and sucrose-pelleted virions were harvested 24 hr post-transfection, analyzed by western blotting, and quantified using an Odyssey imaging system (Li-COR, Inc.). Primary antibodies were rabbit anti-CA and rabbit anti-MA at 1:15,000. HIV-infectious titers were assayed in single-cycle MAGIC assays in P4 cells. Additional experimental details are provided in [von Schwedler et al. \(2003\)](#).

EIAV VLP Production and ALIX Silencing

HeLa M cells (~4 × 10⁵ cells/well, 6-well plate) were transiently transfected (10 μl; FuGene6, Roche Applied Science) with 5 μg of wild-type or p9^{Gag} ΔYP pEV53B EIAV vector (Olsen, 1998). EIAV virus-like particles were harvested 48 hr posttransfection, concentrated through 20% sucrose cushions, and analyzed by western blotting using affinity-purified EIAV anti-CA antibody (1:10,000). For ALIX-depletion experiments, an shRNA targeting human ALIX nucleotides 1765–1783 (Chen et al., 2005) was delivered using the FG12 lentiviral expression vector (Qin et al., 2003; 8 μg/ml polybrene, moi = 15) 24 hr prior to transfection with pEV53B.

Supplemental Data

Supplemental Data include Experimental Procedures, References, five figures, and two tables and can be found with this article online at <http://www.cell.com/cgi/content/full/128/5/841/DC1/>.

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Accession Numbers

Coordinates and data have been deposited into the Protein Data Bank (www.pdb.org; PDB codes 20EV, 20EW, and 20EX).