Activation of the Retroviral Budding Factor ALIX^\dagger\‡

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The cellular ALIX protein functions within the ESCRT pathway to facilitate intraluminal endosomal vesicle formation, the abscission stage of cytokinesis, and enveloped virus budding. Here, we report that the C-terminal proline-rich region (PRR) of ALIX folds back against the upstream domains and auto-inhibits V domain binding to viral late domains. Mutations designed to destabilize the closed conformation of the V domain opened the V domain, increased ALIX membrane association, and enhanced virus budding. These observations support a model in which ALIX activation requires dissociation of the autoinhibitory PRR and opening of the V domain arms.

Retroviral Gag polyproteins contain short sequence motifs, termed “late domains,” that facilitate virus budding by recruiting components of the cellular ESCRT pathway (4, 38). For example, the HIV-1 p6Gag protein contains “PTAP” and “YPXL” late domains (designated by their consensus sequences), that bind directly to the TSG101 and ALIX proteins, respectively (6, 9, 19, 33, 39). ALIX, in turn, binds the CHMP4 subunits of the ESCRT-III complex, resulting in recruitment of the VPS4 ATPase, membrane fission, and virus release (10, 27).

ALIX contains three distinct structural elements: an N-terminal Bro1 domain, a central V domain, and a C-terminal proline-rich region (PRR). The boomerang-shaped Bro1 domain binds CHMP4 proteins (7, 13, 20), the V domain comprises two extended three-helix bundles and binds YPXL late domains (7, 16, 44, 45), and the PRR binds a series of other proteins but is predicted to lack a persistent secondary or tertiary structure (7, 8, 24). Like other ESCRT factors, ALIX must cycle between soluble (inactive) and membrane-associated (active) states. Several lines of evidence suggest that conformational changes accompany (or induce) these transitions. First, recombinant ALIX proteins can form stable monomers and dimers (7, 23), and biochemical evidence suggests that the dimer is the active conformation (5, 7, 23, 29). Second, small and dimers (7, 23), and biochemical evidence suggests that the activation of the Retroviral Budding Factor ALIX requires dissociation of the autoinhibitory PRR and opening of the V domain arms.

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monomeric. The log $I(q)$ versus $q$ SAXS profile for ALIX (open circles) is shown in Fig. 1B. Indirect Fourier transformation of the SAXS profile using the program GIFT (3) yielded the probable atom-pair distance distribution within ALIX $P(r)$ versus $r$ plot (Fig. 1C), the radius of gyration ($R_g$), 44.8 Å, and the maximum linear dimension ($D_{max}$), 155 Å. These structural parameters are very similar to the $R_g$ (45.3 Å) and $D_{max}$ (158 Å) values calculated from the $P(r)$ profile derived from the ALIXBro1-V crystal structure (7). The areas under the $P(r)$ curves are proportional to $I(0)$ and correctly scaled according to the ratios of the square of the molecular masses of the proteins. (D and E) ALIX models that fit the scattering data (red lines in panel B). The Bro1 domain is shown in light blue, the two arms of the V domain are shown in green and blue, respectively, and PRR dummy atoms are shown in magenta. (F) A model in which the PRR projects into solution does not fit the SAXS data (blue line in panel B).

 Isothermal titration calorimetry was performed to test whether the PRR influenced ALIX binding to a high-affinity late-domain peptide from equine infectious anemia virus (EIAV) p9Gag (19TQNLYPDLEIKK31; bold residues contact arm2 of the ALIX V domain) (44). As expected, the control ALIXBro1-V protein bound the EIAV p9Gag peptide with an equilibrium dissociation constant ($K_D$) of 3.6 μM (Fig. 2, black squares), which matches our previous biosensor-based analyses ($K_D$ of 6 μM; see reference 44). In contrast, full-length ALIX did not bind detectably to the EIAV p9Gag peptide under these conditions (open circles). Similarly, the EIAV p9Gag peptide bound approximately 100-fold less tightly to ALIX than to ALIXBro1-V in biosensor binding experiments (data not...
shown). In contrast, the two ALIX constructs bound with similar affinities to a peptide that corresponded to the binding epitope on CHMP4B (292KKKEEDDDEMKELNAGSM222-232), bold residues contact the ALIX Bro1 domain [see reference 20]) (ALIX KD was 112 ± 57 μM, and ALIXBro1-V KD was 75 ± 24 μM [n = 6]). In this respect, our results differ from those of Zhou et al., who observed that the PRR also inhibited CHMP4 binding (48). However, our experiments were performed using pure monomeric ALIX and CHMP4B peptides, whereas theirs were performed with full-length proteins in crude extracts and could have been influenced by additional factors, such as avidity effects resulting from protein oligomerization. Our data indicate that the PRR does not significantly alter the intrinsic affinity of the CHMP4 binding site on the Bro1 domain but strongly inhibits YPXL late-domain binding to arm2 of the V domain.

The two arms of the ALIX V domain are juxtaposed at an acute angle of ~30° in crystal structures of the monomeric protein (termed the “closed” conformation) (7, 16, 44, 45). The suggestion that the linker connecting the two arms functions as a hinge that opens further under some conditions (7) is consistent with SAXS data, which indicate that the two arms are indeed more open in the ALIX dimer (29). We therefore hypothesized that mutations that destabilize the closed conformation might promote ALIX dimerization, membrane association, binding to the oligomeric Gag protein, and virus budding. This idea was tested by examining whether mutation of ALIX Arg649 localized the protein to cellular membranes or stimulated virus release. Arg649 forms an array of hydrogen bonding interactions that connect the three linker strands in the closed conformation, and the Arg649Glu mutation is therefore expected to destabilize the closed conformation of the V domain. Structural parameters derived from SAXS data collected from ALIXBro1-V,R649E (see Table S1 and Figure S2 in the supplemental material) show that there is a mass redistribution in the mutant compared to the ALIXBro1-V crystal structure as indicated by a change in the probable atom-pair distribution (Fig. 1 C, gray diamonds), which extends out to ~190 Å. The Rs of ALIXBro1-V,R649E is ~8 Å greater than the ALIXBro1-V crystal structure (53.5 Å versus 45.3 Å), while the average radius of gyration of cross section (Rs) of the mutant (calculated in PRIMUS) (14) is smaller (Rs of ~12 Å versus ALIXBro1-V of ~14.5 Å). These data show that the Arg649Glu mutation causes ALIXBro1-V to extend and become “thinner,” indicating that the mutation causes the V-domain to occupy a more “open” configuration.

Membrane flotation experiments were performed as previously described (25) to test whether the Arg649Glu mutation enhanced ALIX membrane association. Briefly, transfected 293T cells were collected 6 h posttransfection, washed three times with cold NTE buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA), and suspended in 800 μl NTE buffer containing 6% (wt/vol) sucrose and a protease inhibitor cocktail (Sigma). Cells were disrupted by sonication (Fig. 3A, lane 1, Lysate), and nuclei and protein aggregates were removed by low-speed centrifugation (800 x g for 15 min; resolubilized in 10% triton/ NTE buffer) (Fig. 3A, lane 3, Pellet). ALIX and ALIXR649E remained in the nonpelleted fractions (Fig. 3A, lane 2, Supernatant). This fraction was adjusted to 80% (wt/vol) sucrose in NTE and placed on the bottom of a 14- by 89-mm centrifuge tube (331372; Beckman), and the membrane fractions were “floated” by sedimentation (35,000 rpm for 24 h at 4°C; Beckman SW41 rotor) through layers of 65% sucrose (6 ml) and 10% sucrose (2.5 ml). Fractions containing membrane-bound proteins (4 ml, lane 4) and soluble proteins (5 ml, lane 6) and an intermediate fraction (3 ml, lane 5) were collected, and their protein contents were analyzed by Western blotting. Control soluble (aldolase) and integral membrane (cadherin) proteins concentrated in the soluble and membrane fractions, respectively, as expected (Fig. 3A, rows 3 and 4, respectively). The wild-type FLAG-ALIX protein remained predominantly (79%) in the soluble fraction (Fig. 3A, row 1, compare lanes 4 and 6), whereas most (65%) of the ALIXR649E mutant associated with membranes (Fig. 3A, row 2, compare lanes 4 and 6). Thus, these data indicate that V domain opening activates ALIX for membrane binding.

To test whether the ALIXR649E mutant was also hyperactive in stimulating virus release, we measured the release and infectivity of an HIV-1NL4-3 virus that could not bind TSG101 (HIV-1 ΔPTAP) and was therefore highly dependent upon expression of exogenous ALIX for budding (7, 37). As shown in Fig. 3B and C, ALIXR649E was more potent than the wild-type protein in stimulating virus release and infectivity, particularly when ALIX levels were limiting. For example, viral infectivity was 7-fold higher when cells were transfected with 0.01 μg of the ALIXR649E expression construct (Fig. 3B, inset) even though the two proteins were expressed at equivalent levels (Fig. 3C, panel 3, compare lanes 2 and 8). Similarly, 0.1 μg of the ALIXR649E expression construct stimulated virus release and infectivity to levels that were comparable to those induced by 1.0 μg of the wild-type ALIX expression construct (Fig. 3B, inset; Fig. 3C, panel 3, compare lanes 7 and 11). Thus, the Arg649Glu mutation activates ALIX to facilitate HIV-1 release, further supporting the idea that destabilizing the
closed conformation of the ALIX V domain produces a constitutively active protein.

Together with previous reports (5, 29, 48), our studies support a model in which ALIX is activated to facilitate virus budding through a series of conformational changes that (i) release the PRR from the Bro1-V domains and expose the YPXL late-domain binding site, (ii) open the V domain, (iii) stimulate membrane recruitment of ALIX, and (iv) induce protein dimerization (Fig. 4). These conformational changes are likely to be concerted and mutually reinforcing, because (i) ALIX dimerization appears to require V domain opening (29) and to be regulated by PRR residues (5), (ii) membrane recruitment will tend to increase local ALIX concentrations and thereby drive dimerization, and (iii) ALIX dimerization will tend to promote binding to oligomeric Gag complexes owing to avidity. Thus, all of these effects likely combine to stimulate ALIX recruitment during viral Gag protein assembly at the plasma membrane. Other factors that may also contribute to ALIX activation include ubiquitin association (12), phosphorylation (31), the ubiquitin E3-ligase POSH (1, 36, 42), and factors that bind the PRR, such as CEP55, endophilins, TSG101, ALG-2, PYK2, Src kinases, and the Cbl-SET/CIN85-endophilin complex (5, 7, 18, 21, 22, 30, 32, 33, 36, 40, 41).

In addition to providing a regulatable step in ESCRT complex assembly, ALIX dimerization may nucleate the assembly of two strands of CHMP4, which is thought to form filaments within the necks of budding vesicles (10, 27). During yeast intraluminal endosomal vesicle formation, the ESCRT-II complex performs an analogous function in nucleating the polymerization of two
CHMP4 strands (35). In case, two CHMP4 filaments are formed because the ESCRT-II complex contains two copies of the ESCRT-III binding protein Vps25p (10, 27). Similarly, the mechanism of PRR autoinhibition described here for ALIX is analogous to autoinhibition of the ESCRT-III proteins, whose C-terminal tails also fold back on the body of the protein to prevent protein oligomerization and membrane binding until they are released by binding to upstream factors (2, 15, 17, 43). Once the proteins are opened, the oligomerization domains can polymerize, and the C-terminal tails are free to recruit additional downstream factors. Thus, different ESCRT factors employ common principles to cycle on and off membranes and maintain the sequential protein assembly pathways required for regulated membrane fission.

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