

The multiple personalities of Alix

Greg Odorizzi

Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309-0347, USA
e-mail: odorizzi@colorado.edu

Accepted 1 June 2006

Journal of Cell Science 119, 3025-3032 Published by The Company of Biologists 2006
doi:10.1242/jcs.03072

Summary

Alix is a cytosolic protein in mammalian cells that was originally identified on the basis of its association with pro-apoptotic signaling. More recent evidence has established that Alix has a hand in regulating other cellular mechanisms, including endocytic membrane trafficking and cell adhesion. Although Alix appears to participate directly in these various activities, the role it plays in each process has largely been inferred from the functions of proteins with which it interacts. For example, recruitment of Alix to endosomes is mediated by its N-terminal Bro1

domain, the structure of which was recently solved for its yeast orthologue, Bro1. The diversity of Alix functions is due to its proline-rich C-terminus, which provides multiple protein-binding sites. With this blueprint in hand, we can now ask whether Alix acts simply as an adaptor that links different proteins into networks or, instead, contributes a specific function to distinct molecular machineries.

Key words: Alix, Bro1, MVB, Endocytosis, Apoptosis

Introduction

Proteins that can regulate seemingly disparate mechanisms do so by engaging in multiple distinct protein interaction networks. Such multifunctional activities are characteristic of a protein in mammalian cells known as 'Alix' or 'AIP1'. Both names derive from its original discovery as an interaction partner of ALG-2, a Ca²⁺-binding protein implicated in apoptotic signaling (Missotten et al., 1999; Vito et al., 1999). Here, I use Alix to avoid confusion with the yeast Aip1 protein that promotes severing and capping of actin filaments (Okada et al., 2006). Solid evidence supports a functional link between Alix and apoptosis, but studies have also connected Alix to endocytic membrane trafficking and cytoskeletal remodeling. In addition, several viruses exploit Alix in order to bud from infected cells (Fig. 1).

The ability of Alix to participate in a spectrum of activities stems from its domain architecture (Fig. 2). Its N-terminal 'Bro1 domain' mediates localization to endosomes, a major functional site for Alix; its C-terminal region interacts with the majority of proteins that connect Alix to cellular processes. The Bro1 domain and C-terminal region are linked by a relatively uncharacterized sequence containing two coiled-coil domains. This tripartite domain organization occurs in predicted orthologues of Alix in fungi, plants, worms, flies and fish. More-divergent relatives of Alix also contain the Bro1 domain but bear little resemblance to Alix otherwise and they presumably use the Bro1 domain to associate with endosomes for unrelated purposes.

The roles of Alix have largely been inferred from the functions of proteins with which it interacts. However, relatively little is known regarding the specific molecular functions of Alix in these processes. Below, I summarize the roles that have been proposed for Alix and discuss these in light of the function of yeast Bro1, which is the best characterized orthologue of Alix.

Protein sorting at endosomes

Much of the recent progress in addressing the function of Alix has centered upon its connection to endocytic membrane trafficking. A role for Alix in this process was initially suggested by observations that the loss of Bro1 function in yeast impairs the downregulation of cell-surface proteins that normally undergo endocytosis and delivery to the vacuole, the functional equivalent of the lysosome in higher eukaryotes (Forsberg et al., 2001; Springael et al., 2002). Subsequent characterizations revealed that Bro1 is a member of the 'class E' subset of vacuolar protein sorting (*VPS*) gene products (Nikko et al., 2003; Odorizzi et al., 2003). More than 18 distinct class E Vps proteins have been characterized, all of which are conserved throughout eukaryotes (reviewed by Babst, 2005). Collectively, class E Vps proteins function at the cytosolic face of endosomal membranes to sort integral membrane proteins into luminal vesicles following invagination of the endosomal membrane. Because of their distinctive appearance, endosomal compartments that contain luminal vesicles are commonly referred to as multivesicular bodies (MVBs). In most cases, the luminal vesicles are delivered into the hydrolytic interior of the lysosome after fusion of the MVB perimeter membrane with the lysosomal membrane (Fig. 1) (Futter et al., 1996; Mullock et al., 1998; van Deurs et al., 1995).

Interactions between Alix and the ESCRT machinery

Significant advances in our understanding of the function of Alix have stemmed from characterization of other class E Vps proteins, many of which assemble into hetero-oligomeric endosomal sorting complexes required for transport (ESCRTs), a group of complexes involved in sorting MVB cargoes (reviewed by Hurley and Emr, 2006). Proteins are marked for entry into MVB vesicles by monoubiquitylation of their cytosolic domains, and several of the ESCRTs contain ubiquitin-binding modules that interact directly with

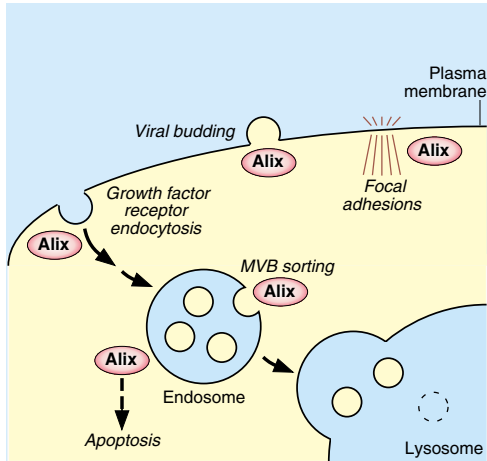


Fig. 1. Overview of the cellular mechanisms involving Alix. For clarity, growth factor receptors and viral components have been omitted, trafficking from the plasma membrane to endosomes is represented by two arrows, and focal adhesions are oversimplified. Fusion of the endosomal/MVB limiting membrane with the lysosome membrane results in the degradation of luminal MVB vesicles by lysosomal hydrolases. Note that the association of Alix with endosomes appears to be important for its role in apoptotic signaling.

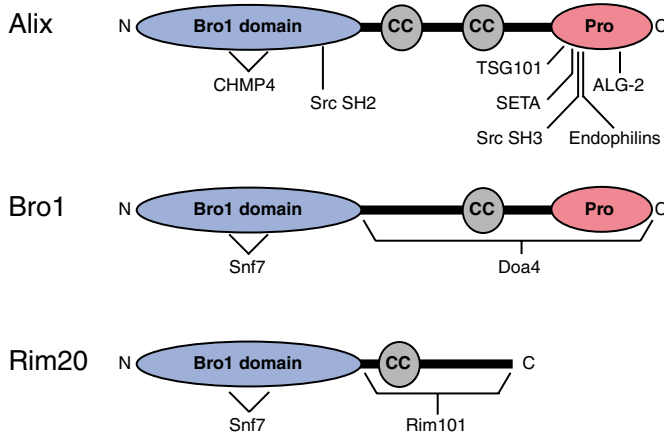


Fig. 2. Schematic diagram depicting the domain organization of Alix and its family members in yeast, Bro1 and Rim20. Alix in humans contains 868 amino acid residues and is 94% identical to mouse Alix, which has 869 residues. Bro1 is similar in length (844 residues) and domain organization, whereas Rim20 is shorter (661 residues) and lacks a C-terminal proline-rich (Pro) region. Based upon the structure of the Bro1 domain in Bro1 (Kim et al., 2005), the Bro1 domains of Alix and Rim20 are predicted to span residues 1-366 and 1-387, respectively. Two putative coiled-coil domains (CC) are located between residues 430-471 and residues 543-583 of Alix, whereas Bro1 and Rim20 are each predicted to have a single CC domain between residues 543-583 (in Bro1) and 386-426 (in Rim20). The proline-rich region of Alix has the majority of protein-binding sites that link it to various cellular mechanisms (see Table 1 for details on specific amino acids involved in protein interactions). Similarly, the proline-rich region of Bro1 is contained within a region required for interaction with Doa4 (Kim et al., 2005). The C-terminal region of Rim20 binds the Rim101 transcription factor (Xu and Mitchell, 2001).

ubiquitylated cargoes (Fig. 3). The ubiquitin E2 variant (UEV) domain in the TSG101 subunit of ESCRT-I in mammalian cells binds not only to ubiquitylated cargo but also to a P[T/S]AP motif in the C-terminal region of Alix (Table 1) (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). Structural analysis indicates that the UEV domain binds ubiquitin and P[T/S]AP sequences through different intermolecular contacts (Pornillos et al., 2002), but we do not know whether TSG101 binds simultaneously to Alix and ubiquitylated cargoes *in vivo*. In fact, the functional significance of the interaction between TSG101 and Alix has yet to be determined. This association does not occur in yeast, because Bro1 lacks a P[T/S]AP sequence and does not directly interact with Vps23, the yeast orthologue of TSG101 (Bowers et al., 2004).

A second point of contact between Alix and the ESCRT machinery is ESCRT-III (Fig. 3). Unlike ESCRT-I, ESCRT-III lacks subunits that contain ubiquitin-binding domains (Hurley and Emr, 2006). Rather than associate with MVB cargoes, ESCRT-III recruits Alix and other class E Vps proteins to endosomes. The ESCRT-III subunit that interacts directly with Alix is CHMP4, three isoforms of which exist in mammalian cells. Alix binds to each CHMP4 isoform (Katoh et al., 2004; Katoh et al., 2003; Martin-Serrano et al., 2003; Peck et al., 2004; Strack et al., 2003; von Schwedler et al., 2003), although CHMP4b is its primary interaction partner (Katoh et al., 2004). Similarly, Bro1 interacts directly with Snf7, the only CHMP4 orthologue in yeast (Kim et al., 2005).

Recent structural analysis of the 'Bro1 domain' of Bro1 has revealed the molecular basis for its interaction with Snf7 (Kim et al., 2005). The Bro1 domain is built around a core of helices with a set of hydrophobic amino acids exposed on one side that comprise the Snf7-binding site (Fig. 4). The interaction between Alix and CHMP4 is likely to be similar because an N-terminal fragment of Alix that encompasses the entire Bro1 domain binds all three CHMP4 isoforms (Katoh et al., 2004; Peck et al., 2004), whereas smaller fragments of the Alix Bro1 domain are unable to bind CHMP4 proteins (Peck et al., 2004). By interacting with the Bro1 domain, Snf7 mediates recruitment of Bro1 to endosomes (Kim et al., 2005; Odorizzi et al., 2003). Immunofluorescence studies suggest that a similar mechanism is responsible for the recruitment of Alix to endosomes because overexpression of CHMP4b causes accumulation at endosomes of both full-length Alix and a truncated form containing the Bro1 domain (Katoh et al., 2003).

The Bro1 domain is a common element in several proteins that localize to endosomes but have no apparent function in protein sorting. Rim20, for example, is the only yeast protein other than Bro1 that contains a Bro1 domain, but Rim20 lacks the proline-rich C-terminal region found in Bro1 and Alix (Fig. 2). Rim20 does not appear to have a role in cargo sorting (Odorizzi et al., 2003). Instead, the association of Rim20 with Snf7 at endosomes results in the recruitment of Rim13, a protease, as well as Rim101, a transcription factor required for cells to grow normally in an alkaline environment (Xu and Mitchell, 2001). As a consequence of their recruitment, Rim13 proteolytically cleaves a C-terminal inhibitory domain from Rim101, thereby enabling Rim101 to be released from endosomes and enter the nucleus (Lamb et al., 2001). The association of Rim20 with Snf7 at endosomes is dependent

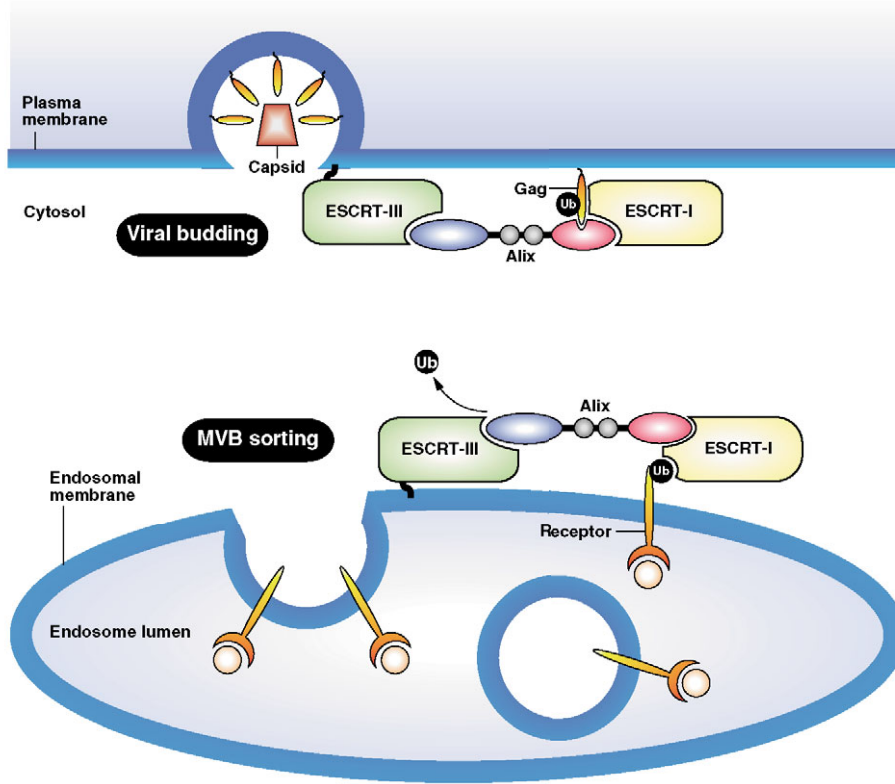


Fig. 3. Alix and its binding partners in ESCRT-mediated protein sorting. For simplicity, components of the ESCRT machinery not known to bind Alix are omitted. Recruitment of Alix to the site of MVB sorting is mediated by the CHMP4 subunit of ESCRT-III, which binds directly to the Bro1 domain. It is not known whether CHMP4 has a similar role in recruiting Alix to the site of viral budding at the plasma membrane, nor is it known whether recruitment of Alix to either endosomes or the plasma membrane is facilitated by the TSG101 subunit of ESCRT-I. The wavy line connecting ESCRT-III to the membrane represents N-myristoylation of CHMP6, another subunit of ESCRT-III. Alix is depicted as a bridging factor between ESCRT-I and ESCRT-III, but whether Alix binds simultaneously to both complexes is not known. Note that several viruses use the ESCRT machinery to bud into endosomes and are then released from infected cells upon fusion of the endosomal membrane with the plasma membrane (reviewed by Morita and Sundquist, 2004). See text for further details. Ub, ubiquitin.

upon alkaline pH because, under acidic growth conditions, Rim20 is exclusively cytosolic, and Rim101 thus remains inactive (Boysen and Mitchell, 2006). Interestingly, Bro1 appears to have a reciprocal dependence upon pH, because its association with endosomes is significantly reduced in alkaline conditions compared with acidic conditions (Boysen and Mitchell, 2006). Although the molecular mechanisms are unclear, the differential affinities of Rim20 and Bro1 for endosomes might be due to a pH-dependent signaling cascade that alters the composition of endosomal membranes; this could result in conditions that are more favorable for either Rim20 or Bro1 to bind Snf7 (Boysen and Mitchell, 2006).

The specific role of Alix in ESCRT-mediated protein sorting has yet to be determined. However, recent work has revealed that, in yeast, Bro1 facilitates deubiquitylation of MVB cargo proteins (Nikko et al., 2003; Odorizzi et al., 2003). Doa4 is the ubiquitin thiolesterase that catalyzes deubiquitylation of cargoes in yeast before the membrane scission event that releases MVB vesicles into the endosome

lumen (Dupre and Haguenaer-Tsapis, 2001; Katzmann et al., 2001; Losko et al., 2001). Bro1 interacts with Doa4 and, in the absence of Bro1, the localization of Doa4 to endosomes does not occur efficiently and cargo deubiquitylation is impaired (Luhtala and Odorizzi, 2004). This indicates that a major function for Bro1 is to recruit Doa4 to the site of MVB cargo sorting.

Whether Alix operates in a similar fashion is not known. In fact, despite its intimate connections with the ESCRT machinery, whether Alix has any role in MVB sorting has been questioned because degradation of the epidermal growth factor receptor (EGFR), long known to be an MVB cargo, is not blocked by RNA silencing of Alix (Cabezas et al., 2005; Schmidt et al., 2005; Schmidt et al., 2004). EGFR degradation, however, is only modestly affected under conditions in which MVB vesicle formation is impaired because the receptor can undergo proteolysis when mislocalized to the lysosomal membrane (Futter et al., 2001; White et al., 2006). Whether EGFR is similarly mislocalized upon silencing of Alix is not

Table 1. Summary of Alix protein-protein interactions

Alix-binding protein	Alix-binding motif	Binding site in Alix	Cellular activity
TSG101 (ESCRT-I)	UEV domain	P ₇₁₇ SAP ₇₂₀	MVB sorting and viral budding
CHMP4* (ESCRT-III)	Unknown	Bro1 domain, Patch 1	MVB sorting and viral budding
Gag [†]	YPX _n L [‡]	Unknown	Viral budding
SETA	SH3 domain	P ₇₄₀ TPAPR ₇₄₅	Growth factor receptor endocytosis; focal adhesion remodeling
Endophilin	SH3 domain	P ₇₅₅ ARPPP ₇₆₁	Growth factor receptor endocytosis
Src	SH2 domain	Phospho-Y ₃₁₉	Growth factor receptor endocytosis; focal adhesion remodeling
Src	SH3 domain	P ₇₅₂ QPPAR ₇₅₇	Growth factor receptor endocytosis; focal adhesion remodeling
ALG-2	Unknown	PGY repeats (aa 802-813)	Apoptosis

*Three CHMP4 isoforms exist in mammalian cells, of which CHMP4b is the predominant Alix-interaction partner. [†]Gag proteins encoded by EIAV, HIV-1, and murine leukemia virus. [‡]X_n indicates one to three unspecified amino acids.

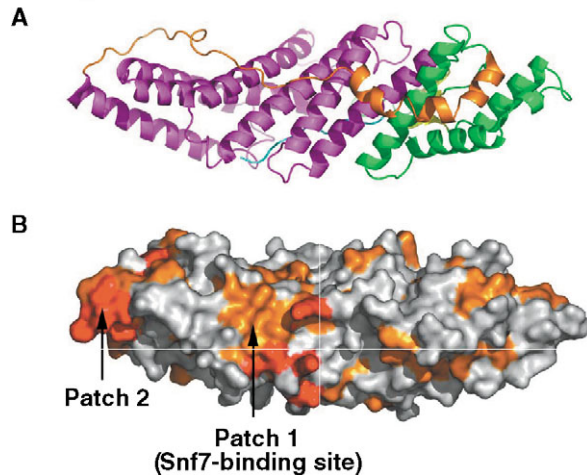


Fig. 4. The Bro1 domain from the yeast Bro1 protein. (A) Ribbon diagram of the Bro1 domain. (B) Molecular surface of the Bro1 domain colored according to residue conservation, orange and red representing amino acids that are similar and identical, respectively, among orthologues of Bro1. Reproduced with permission from Elsevier (Kim et al., 2005).

known. Other recent work, however, has implicated Alix in the control of MVB vesicle formation.

A functional link between Alix and MVB vesicle formation

Evidence connecting Alix to MVB vesicle formation has come from *in vitro* experiments addressing how the multivesicular morphology of endosomes is controlled by 2,2'-dioleoyl lysobisphosphatidic acid (LBPA), an isomer of phosphatidylglycerol enriched in late endosomal membranes of mammalian cells (Kobayashi et al., 2002; Kobayashi et al., 1998). Fluid-phase endocytosis of anti-LBPA antibodies by living cells causes luminal endosomal membranes to adopt a disorganized appearance (Kobayashi et al., 1998), which suggests that MVB dynamics are influenced by the content of LBPA in membrane bilayers. Indeed, synthetic liposomes prepared with LBPA spontaneously form luminal vesicles in a manner analogous to MVB vesicle budding *in vivo* (Matsuo et al., 2004). Remarkably, this LBPA-dependent *in vitro* budding reaction is potently inhibited by recombinant Alix (Matsuo et al., 2004).

Defining the antagonistic role of Alix is difficult because the mechanism by which LBPA stimulates invagination and/or fission of membrane bilayers is unknown. The function of LBPA itself is confusing because it also stimulates liposomes to undergo fusion with one another *in vitro* (Kobayashi et al., 2002). Collectively, these observations have fueled speculation that LBPA destabilizes endosomal membranes, thereby promoting both budding of MVB vesicles and their 'back-fusion' with the limiting endosomal membrane. Back-fusion of MVB vesicles was originally characterized as a mechanism used by antigen-presenting cells to regulate the distribution of major histocompatibility complex class II molecules (Murk et al., 2002), but, in other cell types, pathogens that conceal themselves within MVB vesicles use back-fusion to enter the cytosol (Abrami et al., 2004; Le Blanc et al., 2005). If LBPA

facilitates both MVB vesicle fusion and fission events, Alix might fulfil a regulatory role by serving as a sequestration device to limit the availability of LBPA. Indeed, depletion of Alix *in vivo* reduces the number of acidic late endosomes containing luminal membranes by ~50% (Matsuo et al., 2004), whereas excess amounts of Alix disrupt the back-fusion of MVB vesicles loaded with vesicular stomatitis virus capsids (Le Blanc et al., 2005).

The relationship between Alix and LBPA appears to be unique to higher eukaryotic organisms because LBPA has not been detected in yeast. Moreover, the link between Alix and LBPA is likely to be unrelated to the link between Alix and ESCRT-mediated sorting of MVB cargoes because EGFRs and LBPA are located in distinct populations of MVBs (White et al., 2006). Future work might reveal whether Alix influences vesicle formation in MVBs that mediate EGFR downregulation and/or whether components of the ESCRT machinery function in protein sorting at MVBs containing LBPA.

Enveloped virus budding

The link between Alix and ESCRT-mediated protein sorting is not restricted to endosomes. Many enveloped viruses encode proteins containing sequence motifs termed 'late domains' that bind directly to Alix and other components of the MVB sorting machinery (reviewed by Morita and Sundquist, 2004). These enable viruses to recruit host cell factors for the nonlytic production of virions that bud from the plasma membrane, a process that is topologically equivalent to the budding of vesicles into the MVB lumen (Fig. 3). The best characterized viral late domain that binds to Alix is the YPDL sequence in the p9 domain of the Gag protein of equine infectious anemia virus (EIAV). The production of EIAV virions is strongly inhibited by overexpression of truncated Alix fragments (Martin-Serrano et al., 2003; Strack et al., 2003) or by silencing Alix expression by RNAi (Martin-Serrano et al., 2003). Similarly, Alix binds to a YPAL motif in the Gag protein of murine leukemia virus, the budding of which is impaired upon RNAi-mediated depletion of Alix (Segura-Morales et al., 2005). Interestingly, Alix binds a related motif, YPLTSL, in the p6 domain of Gag encoded by human immunodeficiency virus type 1 (HIV-1), but disruption of Alix function causes only a moderate defect in HIV-1 budding (Martin-Serrano et al., 2003; Strack et al., 2003). This may reflect the fact that HIV-1 p6 also contains a P[T/S]AP consensus sequence, which, as described above, binds the UEV domain of TSG101 from ESCRT-I (Garrus et al., 2001). Indeed, several other viruses contain multiple late domains. Since the late domains from different viruses are in some cases functionally interchangeable, there is clearly some level of redundancy (Morita and Sundquist, 2004).

The YPDL sequence in EIAV p9 also binds to the μ 2 subunit of the AP-2 adaptor complex that directs clathrin-mediated endocytosis (Puffer et al., 1998). Interestingly, simultaneous overexpression (or silencing expression) of both μ 2 and Alix causes significantly stronger inhibition of EIAV budding compared with overexpression of Alix or μ 2 alone (Chen, 2005). EIAV may, therefore, combine both early (AP-2) and late (Alix) endocytic mechanisms to facilitate its assembly and release, but whether components of such distinct stages of endocytic transport directly cooperate in viral budding is unclear.

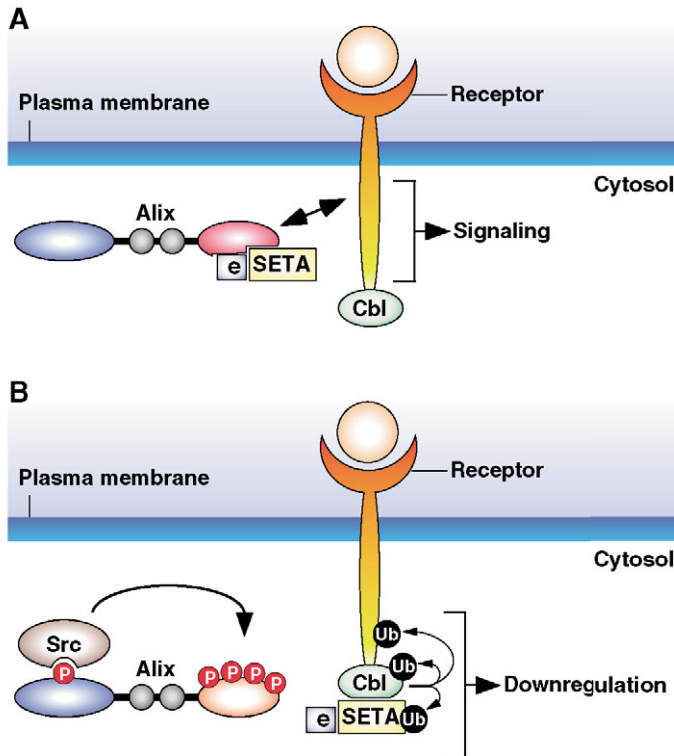


Fig. 5. Model for the negative regulation of EGFR endocytosis by Alix. For simplicity, activated EGFR is represented as a monomer rather than a dimer. (A) Alix inhibits association of the SETA-endophilin complex with Cbl and reduces Cbl-mediated ubiquitylation of EGFR, SETA and Cbl itself (Schmidt et al., 2004). Thus, downregulation of activated EGFR is inhibited (and EGFR signaling is sustained), which could be due to Alix blocking the SETA-endophilin complex from promoting rapid internalization of receptors and/or could be due to Alix facilitating deubiquitylation of Cbl substrates. Note that the non-phosphorylated form of Alix constitutively associates with EGFR (represented by a double-headed arrow) regardless of the activation state of the receptor, although this association appears to occur indirectly. (B) Phosphorylation of Alix by Src prevents Alix from binding SETA (Schmidt et al., 2005), thereby enabling association of Cbl with the SETA-endophilin complex as well as Cbl-mediated ubiquitylation, which promotes downregulation of receptors. e, endophilin; Ub, ubiquitin.

The observations described above, in combination with mutagenic analyses (Vincent et al., 2003), indicate that Alix binds a YP_x_nL consensus sequence (Table 1), but this type of late domain does not represent the only viral connection to Alix. The C protein encoded by Sendai virus (SeV), for example, interacts with Alix but lacks a YP_x_nL sequence (Sakaguchi et al., 2005). The specific residues in C protein that mediate its association with Alix have not been determined, and the way in which SeV exploits Alix may be different from that used by EIAV because C protein is not essential for viral budding. Instead, it accelerates virion production (Sugahara et al., 2004), and this is enhanced by overexpression of Alix (Sakaguchi et al., 2005).

Ubiquitin plays a role in the budding of HIV-1 and many other viruses but appears to be dispensable for EIAV release (Patnaik et al., 2002). However, replacement of the p6 domain

in HIV-1 with the EIAV p9 domain reduces the level of ubiquitylated Gag and, at the same time, facilitates viral budding. Conversely, its replacement with a viral late domain that enhances Gag ubiquitylation reduces the efficiency of HIV-1 release (Martin-Serrano et al., 2004). At least in the context of HIV-1, deubiquitylation might therefore be a key step in the viral budding process. In light of the role that Bro1 has in facilitating MVB cargo deubiquitylation in yeast (Luhtala and Odorizzi, 2004), it seems plausible to suspect that Alix might have a similar function promoting deubiquitylation at the site of viral assembly.

Growth factor receptor endocytosis

Alix appears to have a role in negative regulation of growth factor receptor endocytosis at the plasma membrane, which occurs upstream of ESCRT-mediated protein sorting at endosomes (Fig. 1). Endocytosis is the first step toward long-term downregulation of activated EGFR and precedes its delivery to lysosomes via MVBs. Ubiquitylation of the receptor cytosolic domain triggers its endocytosis and is catalyzed by the Cbl E3 ubiquitin ligase (reviewed by Rubin et al., 2005). Cbl binds to phosphotyrosine residues on EGFR and is activated upon phosphorylation by the receptor. Activated Cbl also recruits an adaptor protein named SETA (also known as CIN85 or Ruk) and can ubiquitylate itself and SETA, which serves to promote rapid internalization of activated receptors (Soubeyran et al., 2002). Endophilins, which constitutively associate with SETA, are also thought to promote endocytosis (Soubeyran et al., 2002).

Alix antagonizes EGFR endocytosis, at least in part, by interfering with the Cbl-SETA interaction (Fig. 5A). A proline-based motif in the C-terminal region of Alix binds directly to an SH3 domain in SETA (Table 1) (Chen et al., 2000; Kowanzetz et al., 2003). Overexpression of Alix prevents the SETA-endophilin complex from binding to Cbl and causes a concomitant reduction in EGFR internalization (Schmidt et al., 2004), presumably because the SETA-endophilin complex is entirely sequestered by excess copies of Alix. A separate proline-based motif in Alix binds to the SH3 domain of endophilins (Table 1) (Chatellard-Causse et al., 2002), which might aid sequestration of the SETA-endophilin complex. Interestingly, overexpression of Alix reduces the ubiquitylation of EGFR, Cbl and SETA (Schmidt et al., 2004), which suggests that Alix also inhibits receptor endocytosis by facilitating deubiquitylation. Indeed, endocytosis of EGFR is enhanced upon siRNA-mediated silencing of either Alix (Schmidt et al., 2004) or AMSH, a ubiquitin thiolesterase that mediates EGFR deubiquitylation (McCullough et al., 2004).

Src regulation of Alix

Whereas Cbl and the SETA-endophilin complex bind only to activated EGFRs, Alix is constitutively associated with the receptor, albeit indirectly (Schmidt et al., 2004). Thus, Alix might sustain EGFR signaling when ligand stimulation is brief and reversible by dephosphorylation. However, during prolonged receptor activation, the ability of Alix to inhibit growth factor receptor endocytosis must be curtailed. Recent work has revealed that Alix is negatively regulated by Src, a protein kinase activated in response to stimulation of EGFR and other receptor tyrosine kinases. In a two-step process, the SH2 domain of Src binds to a phosphotyrosine residue in the

Bro1 domain of Alix; then the SH3 domain of Src binds to yet another proline-based motif in the C-terminal region of Alix (Table 1). As a consequence of this interaction, Src hyperphosphorylates Alix, which has multiple conserved tyrosine residues (Schmidt et al., 2005). Src-mediated hyperphosphorylation interferes with the ability of Alix to interact with SETA and causes it to relocate to the cytosol (Schmidt et al., 2005), thereby neutralizing the negative impact of Alix on receptor endocytosis (Fig. 5B).

Interestingly, the Src SH2 domain-binding site in Alix (Tyr319) corresponds to a tyrosine residue located in a surface-exposed set of hydrophobic residues known as Patch 2 in the Bro1 domain in yeast Bro1 (Fig. 4). In yeast, however, tyrosine phosphorylation is uncommon, and mutation of the corresponding tyrosine residue in Bro1 has no effect on MVB sorting (Kim et al., 2005). Nevertheless, Patch 2 contains the most highly conserved set of amino acids in the Bro1 domain, which suggests that this region performs an important function exploited by Src in mammalian cells and probably also in other metazoans – Xp95, the orthologue of Alix in *Xenopus laevis*, was originally identified as a phosphoprotein regulated by Src activity (Che et al., 1999).

Alix and cell adhesion

The interplay between Alix and SETA and the regulation of Alix by Src are also coupled to the remodeling of focal adhesions – short-lived contact points between the plasma membrane and extracellular substrates (Fig. 1). SETA and Alix both localize to focal adhesions (Chen et al., 2000) and associate with FAK and PYK2 (Schmidt et al., 2003), kinases that regulate the assembly of focal adhesion complexes with cytoskeletal components. Overexpression of SETA enhances cellular adhesion, and this effect is reversed upon co-overexpression of Alix (Schmidt et al., 2003). Overexpression of Src, however, neutralizes the ability of Alix to inhibit focal adhesions (Schmidt et al., 2005). Alix might directly interfere with FAK and PYK2 kinase activity or limit the availability of their substrates. Alternatively, it might interfere with the ability of SETA to promote focal adhesion formation, which would be similar to the way in which Alix restricts SETA from promoting EGFR endocytosis.

Apoptosis

Ironically, the only binding partner of Alix that does not have a functional connection to membrane trafficking is ALG-2, the first protein with which Alix was found to interact (Missotten et al., 1999; Vito et al., 1999). ALG-2 is a member of the penta-EF-hand family of Ca²⁺-binding proteins (Maki et al., 2002) that is required for the induction of apoptosis by a variety of stimuli (Vito et al., 1996). Although the molecular mechanisms involved are not clear, ALG-2 has been linked to both intrinsic and extrinsic apoptotic signaling pathways (Chen and Sytkowski, 2005; Hwang et al., 2002; Rao et al., 2004).

Several lines of evidence suggest that Alix cooperates with ALG-2 to promote apoptosis. Upregulation of endogenous Alix expression correlates with cell death *in vivo* (Blum et al., 2004; Hemming et al., 2004), and overexpression of Alix triggers caspase activation and apoptosis in the absence of pro-apoptotic signals (Mahul-Mellier et al., 2006; Trioulier et al., 2004). Conversely, overexpression of a truncated form of Alix lacking its Bro1 domain protects cells against apoptosis

(Mahul-Mellier et al., 2006; Trioulier et al., 2004; Vito et al., 1999). Association with ALG-2 is required for both the pro-apoptotic function of full-length Alix and the anti-apoptotic ability of the truncated Alix fragment. This interaction requires a PGY repeat sequence in the C-terminal region of Alix (Table 1) (Shibata et al., 2004; Trioulier et al., 2004), the deletion of which renders full-length Alix unable to induce apoptosis and abolishes the protective capacity of truncated Alix (Mahul-Mellier et al., 2006; Trioulier et al., 2004).

Given the anti-apoptotic potential of the truncated form of Alix missing its Bro1 domain, it seems likely that endosomes serve as a platform for signaling pathways that involve the Alix-ALG-2 complex (Fig. 1). Further evidence that endosomes function in this capacity has come from a recent study indicating that the protective function of the truncated Alix fragment is abolished if its ability to bind TSG101 is compromised (Mahul-Mellier et al., 2006). Interestingly, ALG-2 also binds directly to TSG101 (Katoh et al., 2005), but the significance of this interaction remains to be determined.

The pro-apoptotic function of Alix might be related to growth factor receptor signaling. Both cell proliferation and cell death pathways are thought to be activated in response to growth factors to ensure that cells stimulated to proliferate will be eliminated unless additional signals for survival are received (Evan and Littlewood, 1993). In this regard, it would be interesting to know whether growth factor stimulation triggers the assembly of Alix with ALG-2 on endosomes in order to prime cells for entry into apoptosis. Src activates signaling pathways that promote cell survival (Datta et al., 1996; Liu et al., 1998) and might be key to inhibiting apoptotic activation by the Alix-ALG-2 complex because Src-mediated hyperphosphorylation of Alix disrupts its membrane association (Schmidt et al., 2005).

Concluding remarks

The connections between Alix and several cellular processes have become evident during the past few years, but much work remains to be done before we gain a mechanistic understanding of Alix. One issue that must be addressed is whether Alix has a specific molecular activity that it contributes to different cellular machineries (such as the recruitment of ubiquitin thiolesterases) or, instead, functions simply as a multifunctional adaptor that bridges many different types of proteins within networks. In this regard, it is interesting that Alix is abundant within extracellular vesicles known as exosomes, which are luminal MVB vesicles released from cells when MVBs fuse with the plasma membrane rather than lysosomes (Geminard et al., 2004; They et al., 2001). Alix also is enriched in virions that are released from cells under the direction of the HIV-1 Gag protein (Strack et al., 2003; von Schwedler et al., 2003). While it is possible that Alix is packaged as an afterthought into exosomes and virions after it has functioned in the assembly apparatus at the endosome and plasma membrane, respectively, the plenitude of Alix in both types of vesicular carriers indicates that it might have more than an indirect role in their formation.

Finally, Alix might have more widespread influence than is currently recognized. For example, no orthologues of Rim20 are apparent in mammalian cells, which raises the possibility that scaffold assemblies organized in yeast by Rim20 for purposes unrelated to membrane trafficking might be

organized by Alix in mammalian cells. Indeed, recent speculation about its role in apoptosis has suggested that Alix, in combination with ALG-2, could facilitate the recruitment and proteolytic activation of caspases or transcription factors that promote cell death pathways (Sadoul, 2006).

I thank Matthew R. G. Russell and other members of my laboratory for their critical analysis of this manuscript.

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