Do Alix and ALG-2 really control endosomes for better or for worse?

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Models and Speculations

Do Alix and ALG-2 really control endosomes for better or for worse?

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Alix/AIP1 (ALG-2-interacting protein X/apoptosis-linked-gene-2-interacting protein 1) is an adaptor protein that was first described for its capacity to bind to the calcium-binding protein ALG-2 (apoptosis-linked gene 2), the expression of which seemed necessary for cell death. Over-expression of truncated forms of Alix blocks caspase-dependent and -independent mechanisms of cell death. Numerous observations in yeast and in mammalian cells suggest that Alix controls the making of and trafficking through endosomes called MVBs (multivesicular bodies), which are crucial intermediates within the endolysosomal system. In particular, deletion of Bro1, one of the yeast homologues of Alix, leads to an impairment in the function of MVBs, leading to mis-sorting of proteins normally destined to the vacuole. Mammalian Alix may have a similar function and has been shown to bind to lyso(bis)phosphatidic acid, ESCRT (endosomal sorting complex required for transport) proteins, endophilins and CIN85 (Cbl-interacting protein of 85 kDa), which are all main regulators of the endosomal system. EIAV (equine infectious anaemia virus) and HIV late domains use Alix to recruit the ESCRT machinery in order to bud from the cell surface, underscoring the crucial role of the protein in orchestrating membrane deformation. In this review I develop the hypothesis that the normal function of Alix in the endolysosomal system may be deviated by ALG-2 towards a destructive role during active cell death.

Introduction

Alix/AIP1 (ALG-2-interacting protein X/apoptosis-linked-gene-2-interacting protein 1) was first identified in a yeast two-hybrid screen for mouse proteins that interacted with the calcium-binding protein ALG-2 (apoptosis-linked gene 2) (Missotten et al., 1999; Vito et al., 1999). Alix is a cytoplasmic protein, 869 amino acids long in the mouse, with the C-terminal 150 residues rich in proline (32%), tyrosine and glutamine. This PRD (proline-rich domain), which contains several SH3 (Src homology 3) domain-binding motifs [PXXP (Pro-Xaa-Xaa-Pro)] and two WW-binding domains (PPXY), has been shown to bind to endophilins, CIN85 (Cbl-interacting protein of 85 kDa) and Tsg101, whereas the N-terminal region binds to CHMP4 (charged multivesicular body protein 4) (Figure 1A). All of these interacting proteins are known regulators of endolysosomal trafficking. Alix has two homologues in yeast, one involved in the activation of a transcription factor involved in pH adaptation by a cysteine protease, and the other being a regulator of the function of late-endosome intermediates called MVBs (multivesicular bodies). In mammalian cells, Alix has recently been shown to regulate the making of vesicles inside MVBs. The protein is also capable of regulating caspase-dependent and -independent cell death, and this function is under tight control of ALG-2. Here, I will review the known functions of Alix and hypothesize how these may relate to its role in promoting cell death.

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Key words: apoptosis, cell death, endocytosis, endosomal sorting complex required for transport (ESCRT), multivesicular body (MVB).

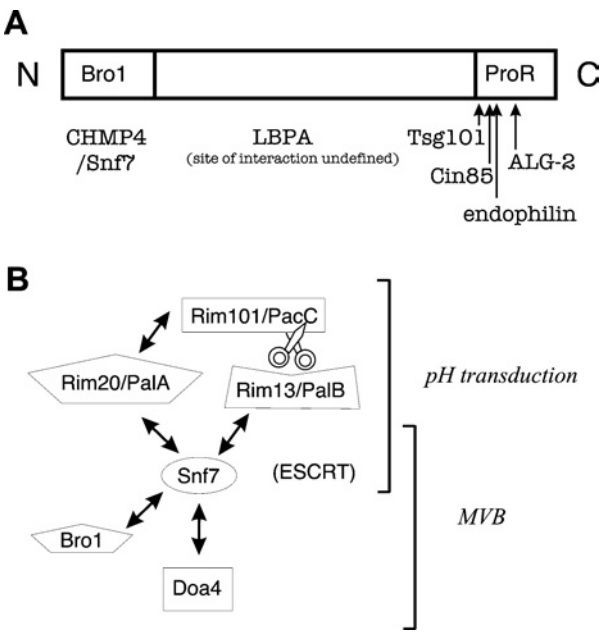
Abbreviations used: AIP1, apoptosis-linked-gene-2-interacting protein 1; ALG-2, apoptosis-linked gene 2; Alix, ALG-2-interacting protein X; CHMP4, charged multivesicular body protein 4; CIN85, Cbl-interacting protein of 85 kDa; EGF, epidermal growth factor; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; ILV, intralumenal vesicle; Lamp1, lysosome-associated membrane protein 1; LBPA, lyso(bis)phosphatidic acid; MVB, multivesicular body; PRD, proline-rich domain; SH3, Src homology 3; Vps, vacuolar protein sorting.

Alix function revealed by fungi

Alix is highly conserved throughout species and has two homologues in *Saccharomyces cerevisiae*, Rim20 and Bro1. Rim20 was first described in *Aspergillus nidulans* as PalA, which is part of a signalling pathway for changes in pH that is conserved throughout fungi. Another central player in this pathway is the

Figure 1 | Alix complexes in mammals and fungi

(**A**) Representation of mammalian Alix and sites of binding with some of the interactors, as described in the text. The interaction site of LBPA has not been mapped; that of CHMP4 has only been estimated to be within the first 160 amino acids, which comprise the Bro1 domain. (**B**) Interactions between Alix homologues in *S. cerevisiae* (Rim 20 and Bro1) and in *A. nidulans* (PalA) and proteins regulating the pH transduction and the MVB pathways. As described in the text, Rim13/PalB belongs to the calpain cysteine protease family, Rim101/PacC is a transcription factor activated after cleavage by Rim13/PalB. Snf7 is a protein of the ESCRT-III complex. Doa4 is an ubiquitin thiolesterase. Interactions are indicated by double-ended arrows.



transcription factor PacC, which acts positively on genes expressed in alkaline conditions and negatively on genes expressed in acid conditions (Arst and Penalva, 2003) (Figure 1B). PalA/Rim20 binds directly to PacC, allowing its proteolytic activation by the cysteine protease PalB in response to ambient alkalination. In yeast, Rim20 was shown to bind to a protein called Snf7 through its N-terminal 160 amino acids, a region highly conserved throughout species which defines the Bro1 domain; this interaction is required for the cleavage of PacC/Rim101 by PalB/Rim13 (Xu and Mitchell, 2001) (Figure 1B).

Yeast two-hybrid analysis has also demonstrated that an interaction occurs between Snf7 and PalB/Rim13 (Ito et al., 2001). One presenr model is that PalA/Rim20 forms a scaffold together with Snf7, bringing the protease PalB/Rim13 into close proximity with its substrate PacC/Rim101 (Xu and Mitchell, 2001). No protein has been identified as a PacC/Rim101 homologue in higher eukaryotes, but PalB/Rim13 is a member of the calpain large-subunit family of cysteine proteases, albeit lacking the penta-EF-hand motif. It is homologous with mammalian calpain-7 (also known as PalBH), the function of which is not known (Futai et al., 2001).

Snf7/Vps32 is a member of the class E Vps (vacuolar protein sorting) family. Mutations in class E *VPS* genes impair the sorting of transmembrane proteins which are being trafficked from the Golgi or from the plasma membrane to the vacuole, and lead to the accumulation of aberrant multilamellar endosomes known as class E compartments. It has been shown that some of the class E Vps proteins associate on endosomes into three large hetero-oligomeric complexes, called ESCRT-I (endosomal sorting complex required for transport I), ESCRT-II and ESCRT-III, which deform the limiting membrane of endosomes into vesicles budding inside these organelles (Katzmann et al., 2002; Raiborg et al., 2003). This leads to the formation of MVBs filled with ILVs (intralumenal vesicles). The MVBs then fuse with the equivalent of the lysosome in yeast, the vacuole, delivering the ILVs and their cargoes to their end station (Gruenberg and Stenmark, 2004).

Bro1, the second *S. cerevisiae* Alix homologue, is allelic to *VPS31*, another class E *VPS* gene (Odorizzi et al., 2003). Like Rim20, Bro1 binds to Snf7/Vps32, which is part of ESCRT III. This allows recruitment of Bro1 on to endosomes, and Bro1 in turn recruits the ubiquitin thiolesterase Doa4 (Luhtala and Odorizzi, 2004). De-ubiquitination is obviously essential for normal endosome traffic, since over-expression of Doa4 reverses the abnormal endosomal morphology seen in Bro1 mutants, thereby restoring normal MVB function. Interestingly, Bro1 is genetically linked to the HECT (homologous with E6-AP C-terminus) domain-containing ubiquitin ligase Rsp5, suggesting that it also interacts with this enzyme which regulates sorting of cargoes through MVBs (Springael et al., 2002; Katzmann et al., 2004). The sequential association of ESCRT complexes is catalysed by the ubiquitinated intracellular domain of transmembrane proteins. These latter are collected, after

de-ubiquitination, in the membranes of ILVs and will end up in the vacuole. This together may explain why Bro1, which may regulate cycles of ubiquitination/de-ubiquitination, is crucial for sorting proteins inside MVBs (Nikko et al., 2003; Odorizzi et al., 2003).

The functional relationship between Rim20p and Bro1 is still unclear. Bro1 functions in the MVB pathway, but not in the Rim101p pathway, whereas Rim20p does not function in the MVB pathway (Xu et al., 2004). It is of note that, even though the two yeast proteins are only 21% identical (158/723), Alix is their closest mammalian homologue (22% identity with Bro1 and 26% with Rim20), suggesting that in higher eukaryotes Alix is the protein with the functions of both Rim20p and Bro1p, or that one of the functions is specific to yeast.

Mammalian Alix may modulate MVB trafficking

Recently, several studies have characterized mammalian homologues of ESCRT proteins and described their mutual interactions. In particular, Alix was shown to interact with CHMP4 proteins, which are homologous with the yeast ESCRT-III protein, Snf7, and with Tsg101, a protein from ESCRT-I, through its Bro1 and PRD domains respectively (Katoh et al., 2003; Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003).

Such interactions may be crucial for normal MVB biogenesis, since the latter was shown to be impaired in HeLa cells depleted of Alix by siRNA (small interfering RNA). In these cells, LBPA [lyso-(bis)phosphatidic acid] staining of Lamp1 (lysosome-associated membrane protein 1)-positive late endosomes was strongly decreased, and electron microscopy revealed a severe depletion of ILVs inside these endosomes (Matsuo et al., 2004). LBPA is a phospholipid highly concentrated in ILVs and plays a role in trafficking through late endosomes, as first demonstrated by the finding that endocytosed antibodies against LBPA block this step. They also cause cholesterol accumulation that mimicks the cholesterol-storage disorders seen in Niemann–Pick disease, underscoring the role of MVBs as a dispatching platform for intracellular cholesterol (Kobayashi et al., 1999). A crucial role for LBPA in endosome maturation was further suggested by Matsuo et al. (2004), who observed that ILVs spontaneously accumulated inside liposomes that contain LBPA, provided that the inside pH was decreased to 5.0 while the exterior was maintained at pH 7.0. This pH difference is similar to that detected between the cytosol and the lumen of endosomes. After incubation of liposomes with cytosol, five proteins were selectively found to be recruited on to LBPA liposomes, but not on to control liposomes, and one protein was identified by tandem mass spectrometry as Alix. Incubation of LBPA-liposomes with recombinant Alix blocked the formation of multivesicular liposomes, whereas depletion of Alix from the cytosol favoured the accumulation of vesicles. Another phospholipid binding protein, annexin II, only partially decreased the amount of multivesicular liposomes (Matsuo et al., 2004). These experiments suggest that LBPA enrichment in endosomes allows the vesiculation process to occur, giving rise to MVBs, and define Alix as a specific modulator of this step.

In addition to binding to LBPA and ESCRT, Alix could also regulate vesiculation of the MVBs through interaction with endophilins. Indeed, we showed that the Alix PRD binds the SH3 domain of endophilins A1, A2 and A3 at a site (P^{748}PAKPQPPARPPPP^{761}) which contains a PXRPPPP consensus sequence in common with other endophilin interactors (Chatellard-Causse et al., 2002). The region of endophilin interaction is distinct from the ALG-2 interacting domain, and both proteins can interact simultaneously with Alix (Shibata et al., 2004). Endophilins type A were originally described as regulators of clathrin-mediated endocytosis, possibly through their interaction with the phosphoinositide 5-phosphatase synaptojanin (Slepnev and De Camilli, 2000; Song and Zinsmaier, 2003). Endophilins have been reported to have lysophosphatidic acid acyltransferase activity; hence they would convert lysophosphatidic acid and acyl-CoA into phosphatidic acid (Schmidt et al., 1999). It has been suggested that transformation of an inverted cone-shaped lysophosphatidic acid into a cone-shaped phosphatidic acid locally modifies the membrane curvature during endocytosis to allow completion of a membrane vesicle (Huttner and Schmidt, 2000). Another remarkable feature of endophilins is the presence of a BAR (Bin/amphiphysin/Rvs) domain formed of curved 'banana-shaped' dimers, which interact with lipid bilayers thereby deforming membranes (Farsad

et al., 2001; Zimmerberg and McLaughlin, 2004). Endophilin binding could therefore generate regions of negative curvature at the neck of the budding vesicle and thereby help its release inside the endosome. Recently, Angers et al. (2004) showed that the SH3 domain of endophilins also binds Itch/AIP4, a HECT domain-containing ubiquitin ligase of the Nedd4/Rps5 family. They observed that Itch localized on the *trans*-Golgi network and on endosomes, whereas endophilins relocalized from the cytosol to endosomes 10 min after EGF (epidermal growth factor) stimulation and became ubiquinated.

Alix may also deform membranes, since we observed that expression of several deletion mutants of Alix leads to accumulation of numerous small perinuclear tubulo-vesicular structures. This is, for example, the case for Alix-CT, a mutant lacking the N-terminal half of the protein. When Alix-CT was co-expressed with endophilin A1, these vesicular structures were transformed into a few very large ones. Interestingly, endophilin over-expression alone did not induce vacuolization, and SH3-deleted endophilin did not enhance the effect of Alix-CT. Co-localization experiments did not reveal the identity of the deformed intracellular compartments. In particular, neither EEA1, which stains early endosomes, nor Lamp1 or anti-LBPA antibody, which stain late endosomes and ILV respectively, were affected by Alix-CT overexpression (Chatellard-Causse et al., 2002). One explanation for why endosomes are apparently unaffected by Alix-CT may be that it lacks the Bro1 domain which allows interaction with CHMP4.

The PRD of Alix also interacts with another scaffold protein, CIN85/SETA/Ruk, involved in endocytosis of ubiquinated tyrosine kinase receptors (Dikic, 2002). CIN85 was first characterized as an interactor of the RING domain-containing ubiquitin ligase Cbl, which mono-ubiquitinates the EGF and c-Met receptors (Petrelli et al., 2002; Soubeyran et al., 2002). CIN85 participates in endocytosis and lysosomal degradation of these receptors through binding to endophilins. Since Alix interacts with main regulators of endocytosis (CIN85 and endophilin) and with ESCRT proteins (Tsg101 and CHMP4), it is ideally suited to accompany and regulate endocytosed tyrosine kinase receptors through endosomes down to lysosomes. In agreement with this, Schmidt et al. (2004) recently showed that increasing the cellular concentration of Alix inhibits the interaction of Cbl with CIN85, decreases the level of ubiquitination of EGF receptor, CIN85 and Cbl, and limits endocytosis of the receptor. However, the effect was only limited to internalization; the authors could not demonstrate any effect of Alix expression on the degradation of the EGF receptor (Schmidt et al., 2004), a lack of effect that we have also observed (J. Fauré and R. Sadoul, unpublished data).

Enveloped viruses know what Alix is good for . . .

Vesicular budding away from the cytosol towards the lumen of the endosome is topologically equivalent to viral budding from the cell surface. This may explain why Gag proteins from membrane-containing viruses, which have a central role in virion assembly at the plasma membrane, bind to and thereby recruit proteins that normally function in MVB biogenesis (Morita and Sundquist, 2004). The Gag motifs involved in the late phases of budding are PT/SAP which binds to Tsg101 [as in the case for HIV-1, HIV-2, FIV (feline immunodeficiency virus), HTLV-I (human T-cell leukaemia virus I), HTLV-II, BLV (bovine leukaemia virus), RSV (respiratory syncytial virus) and MPMV (Mason–Pfizer monkey virus)], $YP(X)_nL$ binding to Alix [HIV-1, RSV and EIAV (equine infectious anaemia virus)] and PPXY binding to Nedd4 (HTLV-I, HTLV-II, BLV, RSV and MPMV). Nedd4 is a HECT domain-containing E3 ubiquitin ligase homologous with Itch and with yeast Rsp5 (see above), and has been shown to play a critical role in membrane protein internalization (Ingham et al., 2004). In the case of Mo-MLV (Moloney murine leukaemia virus), the Gag protein binds to Nedd4, Tsg101, Alix and endophilins, which all influence budding of the virus (Wang et al., 2003; Segura-Morales et al., 2005). It is of note that among all the proteins involved in the endosomal sorting pathway only four have been shown to bind to Gag proteins, despite the large variety of retroviruses. The three late-domain motifs are found in eukaryotes and are involved in Alix interactions with endogenous proteins: Alix PRD contains a PSAP motif able to bind to Tsg101 and two PPXY motifs, which could potentially bind Nedd4 (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003); in *S. cerevisiae* and *A. nidulans* two YPXL/I motifs of Rim101p/PacC are recognized by Rim20p/PalA/Alix (Vincent et al., 2003).

In macrophages, HIV-1 was shown to bud inside MVBs (Pelchen-Matthews et al., 2003). The virus exits the cell when MVBs fuse with the plasma membrane and release the ILVs, which become exocytosed vesicles or exosomes. Exosome release has been demonstrated in a number of cell types. Some of the proteins specifically enriched in exosomal fractions are those of the MVB machinery, such as Alix and Tsg101 which are also found in virions (Thery et al., 2001; Pelchen-Matthews et al., 2004).

ALG-2: a bad influence on Alix?

Even though cysteine proteases of the caspase family are central players in apoptosis, in particular, in driving the destruction of the nucleus, it is clear that other mechanisms are also responsible for cell death. Indeed, in many cells caspase inhibition has no detectable effect on cell survival, but blocks nuclear destruction and reveals an autophagic destruction of the cytoplasm (Bursch, 2001; Lockshin and Zakeri, 2004). In the central nervous system, calcium-induced cell death, due to glutamate toxicity, induces a massive increase of endolysosomal activity, reflected by an up-regulation of autophagy and of endocytosis (Borsello et al., 2003). In the last part of this review I will develop the argument that Alix and ALG-2 may play a role in regulating both caspase-activation and the endolysosomal system necessary for the active destruction of the cell.

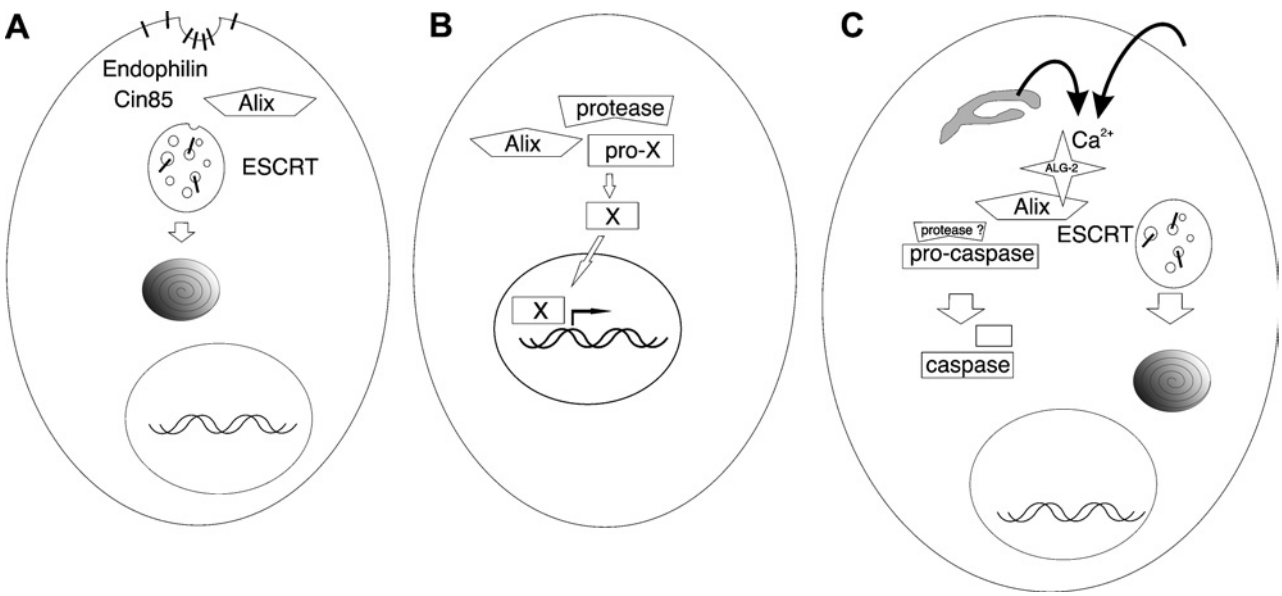
ALG-2 was first described in a functional screen in a T-cell line as a protein whose expression is required for T-cell receptor-, Fas- and glucocorticoid-induced apoptosis (Vito et al., 1996). However, the protection due to the absence of ALG-2 was not observed in T-cells isolated from ALG-2 knockout mice, which exhibit normal T-cell development and function, suggesting that other functionally redundant proteins might exist in mammalian cells (Jang et al., 2002). ALG-2 is a cytosolic 28 kDa protein that forms dimers and belongs to the PEF family. These proteins have five repeats of the EF-hand motif (EF1–EF5) making up the PEF domain (Maki et al., 2002). Another member of the PEF family is the Ca^{2+}-dependent protease calpain, from which the first PEF domain structure was determined. ALG-2 can bind calcium on EF1, EF3 and EF5, and calcium binding is required for its interaction with Alix (Jia et al., 2001). We demonstrated that ALG-2 binding requires a PXY repeat P^{802}PYPTYPGYPGYPGY^{813} in the PRD (Trioulier et al., 2004). Interestingly, phosphorylation of this peptide is regulated by PYK2 (F. Strappazzon, J Fauré and R. Sadoul, unpublished data), a tyrosine kinase known to interact with Alix and ALG-2 (Schmidt et al., 2005). This may be a way of regulating ALG-2 binding, since Shibata et al. (2004) have recently shown that each tyrosine residue of this repeat is critical for binding.

Up-regulation of Alix expression *in vivo* was seen to correlate with cell death, for example, in neurons of the rat hippocampus following epileptic seizures due to intra-peritoneal injection of kainic acid. The hippocampal regions most affected by the sustained rise in Alix expression were those undergoing severe neuronal loss, which is known to be caused by calcium entry through glutamate receptors and to be accompanied by massive up-regulation of endocytosis and autophagy (Hemming et al., 2004). Alix was also augmented in the degenerating striatum of rats chronically intoxicated by 3-nitropropionic acid. This experimental paradigm is a model of Huntington's disease as it reproduces many features of the disease, including behavioural alterations and cortico-striatal degeneration (Blum et al., 2004).

Alix over-expression has been reported to induce detachment of cells from their substrate and to enhance detachment-induced death (anoikis) of HeLa cells (Wu et al., 2002). Over-expresssion of a mutant lacking the N-terminal region of Alix was, in turn, shown to block death of HeLa cells induced by serum starvation (Vito et al., 1999). We observed that Alix over-expression is sufficient to induce apoptosis in neurons. Caspase activation by Alix followed by cell death was demonstrated upon over-expression of the protein in post-mitotic cerebellar granule neurons cultured in high extracellular potassium (Trioulier et al., 2004). In the chick embryo, enforced expression of Alix by electroporation of the neural tube with an expression plasmid, led to the appearance of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-positive cells on the electroporated side (Fraboulet et al., 2003; A.L. Mahul, S. Fraboulet and R. Sadoul, unpublished data). The pro-apoptotic effect of Alix is strictly dependent on ALG-2 binding, since we observed, *in ovo* and *in vitro*, that Alix without the 12-amino-acid long PXY repeat necessary for ALG-2 binding has no deleterious effect on cell survival. On the other

Figure 2 | Hypothetical mechanisms which could explain how Alix influences neuronal death

The three hypotheses are discussed further in the text. (**A**) Alix modulates endocytosis of death or of survival receptors due to its interaction with endophilins, CIN85 or ESCRT. (**B**) By anology with Rim20, Alix is used as a scaffold protein allowing cleavage of an unknown transcription activator ('X') precursor regulating cell death. (**C**) A rise in cytosolic calcium (entering from the extracellular space or leaking out of intracellular stores) allows the interaction of ALG-2 with Alix, which in turn drives the formation of a complex leading to activation of caspases. On the other hand, Alix recruits the ESCRT machinery in order to dismantle the cytoplasm inside late endosomes.



hand, expression of Alix-CT in cultured cerebellar neurons or in chick motoneurons *in ovo* could block caspase activation and cell death, suggesting that this truncated version of Alix acts as a dominant negative. Again, the effect of Alix-CT was abolished by removal of the ALG-2-binding site (A.L. Mahul, S. Fraboulet and R. Sadoul, unpublished data). Therefore, the molecular mechanisms in which Alix/ALG2 intervene seem to be central for cell death *in vitro* and *in vivo*.

In cerebellar granule cell cultures, neurons survive in the absence of growth factors, when they are depolarized by a high extracellular concentration of KCl (25 mM), which allows a sustained elevation of calcium concentration in the cytosol. Death occurs soon after the culture medium is changed to 5 mM KCl. It has all the features of apoptosis, with activation of caspases and nuclear pyknosis and fragmentation. However, several laboratories, including ours, have shown that inhibition of caspases blocks nuclear fragmentation without allowing neuronal survival in low potassium concentrations (Trioulier et al., 2004). This demonstrates that both caspase-dependent and -independent mechanisms contribute to the cell destruction. The fact that Alix-CT not only blocks caspase activation, but also allows neuronal survival, suggests that the Alix–ALG-2 pair may control a signalling step or an execution step common to caspase-independent and -dependent programs of neuronal death. As in other cell types, neuronal apoptosis can be induced by a number of pathways, and the role of Alix in MVB function suggests that its over-expression perturbs endosomal traffic of cell survival or death receptors (Figure 2A). As cerebellar neurons, in which we demonstrated the pro-apoptotic effect, survive in absence of growth factors, it is unlikely that the deleterious activity of Alix is simply due to a modification of signalling by the receptor tyrosine kinases. Furthermore, removal of survival factors from neuronal cultures leads to activation of stress kinases, whereas we found no activation of ERK (extracellular-signal-regulated kinase) or of JNK (c-Jun N-terminal kinase) in cultured neurons dying from Alix over-expression. It is also noteworthy

that Alix-CT without the binding sites for endophilin or CIN85, which possibly link Alix to endocytosis of tyrosine kinase receptors, has the same anti-cell death activity as Alix-CT (Y. Trioulier, S. Torch, F. Strappazzon, J.M. Verna and R. Sadoul, unpublished data). Finally, we have recently observed that over-expression of Alix-CT is capable of blocking the death of motoneurons *in ovo,* at a stage when their survival is not dependent of neurotrophins (A.L. Mahul, S. Fraboulet and R. Sadoul, unpublished data).

As a parallel to PalA/Rim20 in *A. nidulans* and *S. cerevisiae*, one may also hypothesize that Alix may control the processing and activation of a transcription factor necessary for cell death. Indeed, neuronal death, in particular that of cerbellar neurons induced by potassium deprivation, requires transcription to occur (Figure 2B).

Another possible explanation for the cell death-inducing activity of Alix is that it may be part of the caspase execution programme controlled by calcium. Indeed, Rao et al. (2004) have shown that ALG-2 is necessary for caspases to be activated following an abnormal rise in cytosolic calcium. Regardless of the type of death signal, caspase activation requires formation of molecular complexes which allow protease cleavage of caspase zymogen into active caspases. The best characterized caspase activation pathway is that triggered by the mitochondrial release of cytochrome *c* and its subsequent binding to Apaf-1 which serves as an activating platform for pro-caspase-9 (Ferraro et al., 2003). In turn, activated caspase-9 cleaves and activates the downstream caspase-3, which cleaves numerous substrates thereby dismantling the cell. Apoptosis induced by stress of the ER (endoplasmic reticulum) also leads to caspase-9 activation, but requires a high-molecular-mass complex containing the ER-based caspase-12 zymogen and not cytochrome *c* or Apaf-1 (Morishima et al., 2002; Rao et al., 2001). ALG-2 was shown to co-precipitate with caspase-12, and *Apaf-1*$^{-/-}$ cells depleted of ALG-2 were protected against thapsigargin, which induces a rise in cytosolic calcium, but not against other ER stressors, such as tunicamycin or brefeldin A (Rao et al., 2004). Given the tight calcium-dependent Alix–ALG-2 interaction, it is reasonable to hypothesize that Alix is present in the caspase-12 complex. Interestingly, this caspase can be cleaved and activated by calpain (Nakagawa and Yuan, 2000), and we have recently shown that a calpain inhibitor, CI-II, partially inhibits caspase-3 activation in potassium-depleted neurons (Y. Trioulier, S. Torch, R. Sadoul and J.M. Verna, unpublished data). By analogy with Rim20p/PalA promoting cleavage of Rim101p/PacC by the calpain-related protease Rim13/PalB, Alix could serve as a scaffold protein presenting caspase-12, or an equivalent caspase, as a substrate to calpain (Nakagawa and Yuan, 2000) (Figure 2C). The ability of Alix to bind strongly and specifically to YPXL/I motifs also raises the possibility that it might recruit other substrates containing this motif in the ALG-2–calpain complex for proteolysis.

In addition to regulating caspase-activation, ALG-2 could bind to Alix in order to control the ESCRT machinery and thereby the endolysosomal cytoplasmic destruction necessary for cell death (Figure 2C). Indeed, over-expression of an Alix mutant defective for CHMP4 interaction (Bro1 deletion) has been shown to block viral budding, and this was interpreted as being due to blockage of the ESCRT machinery (Strack et al., 2003). Over-expression of Alix-CT, which lacks the Bro1 domain but still binds to Tsg101 (ESCRT-I), should also perturb the normal function of MVBs. This perturbation could be reflected by the abnormal cytoplasmic vacuolization seen in Alix-CT-expressing cells and suggests that ESCRT proteins could participate in cell death.

Yu et al. (2004) have shown the close link between the endolysosomal system and caspases by demonstrating that the autophagic cell-death programme, controlled by the protein beclin-1, is regulated by caspase-8. Also, in sympathetic neurons, inhibition of autophagy by 3-methyladenine is neuroprotective, correlating with inhibition of cytochrome *c* release from mitochondria and prevention of caspase activation (Xue et al., 1999). Our current hypothesis is that ALG-2 reacts to abnormal calcium fluctuations by binding to Alix and thereby drives caspase activation, as well as part of the late endosomal machinery. This would allow the destruction of the cytoplasm and elimination of the protease products by hydrolysis inside lysosomes or by excretion in exosomes. This double game played by Alix in both caspase activation and control of endosomal traffic could be underscored by our observation that, in some neurons, activated caspases are detected inside the Alix-CT-induced vacuoles (Trioulier et al., 2004). Another link is that observed by de Gassart et al. (2003)

showing the presence of activated caspase-3 in exosomes secreted by Daudi cells.

Many more observations will be required to give a definitive judgement in the Alix/ALG-2 affair, thereby answering the question: did they really mean to kill the cell or did they only want to steal its endosomes?

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