Alix (ALG-2-interacting Protein X), a Protein Involved in Apoptosis, Binds to Endophilins and Induces Cytoplasmic Vacuolization

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ALG-2-interacting protein X (Alix), also known as AIP1, is a cytoplasmic protein ubiquitously expressed and concentrated in phagosomes and exosomes. Alix may regulate apoptosis since it binds apoptosis-linked gene 2 (ALG-2), a Ca²⁺-binding protein necessary for cell death, and also overexpression of its C-terminal half (Alix-CT) blocks death induced by several stimuli. This part of Alix contains a long proline-rich domain containing several potential SH3-binding sites. Using Alix as bait in a yeast two-hybrid system to screen a mouse brain library, we have found that SH3p4, SH3p8, and SH3p13, collectively known as endophilins, bind to Alix. Co-immunoprecipitations and overlay experiments allowed us to demonstrate that endophilins bind to Alix-CT through an SH3/proline-rich domain interaction. We have narrowed the region of Alix interacting with endophilins down to 14 amino acids containing a PXRP PPP consensus sequence, also present in synaptojanin and germinal center kinase-like kinase, allowing their interaction to endophilins. We further show that overexpression of Alix-CT, which blocks cell death, leads to cytoplasmic vacuolization into tubulo-vesicular structures delineated by Alix-CT. This vacuolization phenomenon is greatly enhanced upon co-expression with endophilins and may be part of the protecting mechanism afforded by Alix-CT.

Alix¹/AIP1 was first identified as a protein interacting with the calcium-binding protein ALG-2 (apoptosis-linked gene 2), which seems necessary for cell death (1, 2). Inhibiting ALG-2 expression protects cells from death induced by several stimuli (3), an effect similar to that seen upon overexpression of the C-terminal half of Alix-CT, which contains the ALG-2-interacting domain (4). Even though Alix and ALG-2 functions remain obscure, these observations have suggested that both proteins participate in the cell death program.

Alix is a 869-amino acid long cytoplasmic protein that is broadly conserved throughout species. Rim 20p, a homologue of Alix in Saccharomyces cerevisiae, was recently shown to interact with a transcription factor (Rim 101p) and, through SNF7p, with a cysteine protease (Rim 13p), thereby allowing cleavage of Rim 101p (5). SNF7p/VPS32p is involved in endosomal trafficking (5, 6). Alix possesses no obvious enzymatic signature, but its last 150 amino acids are particularly rich in proline (32%), tyrosine, and glutamine residues and contain several Src homology domain 3 (SH3) binding motives (PXXP) and two WW binding domains (PPXY). This 150-amino acid-long proline-rich domain (PRD) interacts with the second SH3 domain of SETA (SH3 domain expressed in tumorigenic astrocytes), also described as Ruk (7–9). Expression of the SETA gene is associated with tumorigenic state in astrocytes. Overexpression of SETA proteins capable of binding Alix sensitized astrocytes to UV light-induced cell death, whereas parts of SETA not binding to Alix had no effect (7). Ruk was described as an adapter protein, forming complexes with the p85α regulatory subunit of the class IA phosphatidylinositol 3-kinase, thereby inhibiting the phosphatidylinositol 3-kinase activity of the enzyme. This activity may explain how overexpressing SETA/Ruk induced apoptosis of cultured primary neurons from the peripheral nervous system (8).

Because the interaction between Alix and SETA/Ruk further suggested a role for Alix in controlling cell death, we searched for pathways that may be regulated by Alix. As a first step we screened for Alix-interacting proteins using a yeast two-hybrid method. We found that Alix interacts with 3 proteins, SH3p4, SH3p8, and SH3p13, which all share almost identical SH3 domains and are known as endophilins or EEN proteins (for review, see Ref. 10). These proteins are known to regulate membrane shape during endocytosis, possibly through their lysophosphatidic acid acyltransferase activity (11) or by regulating enzymes like synaptojanin and dynamin with which they interact through their SH3 domains (12, 13). We narrowed the site of interaction on Alix down to a 14-mer peptide, which shows strong homologies to endophilin binding regions of synaptojanin and of the germinal center kinase-like kinase (14). We also observed that, when overexpressed, the C-terminal half of Alix (Alix-CT), which is known to block cell death, induces the formation of, and accumulates around, a tubulo-vesicular cytoplasmic compartment containing endoplasmic reticulum resident proteins. When the same Alix-CT is co-expressed with endophilins, the vacuoles/tubules are drastically enlarged and can even form very large spherical vacuoles with a diameter of up to several microns.

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The abbreviations used are: Alix, ALG-2-interacting protein X; ALG-2, apoptosis-linked gene 2; AIP1, ALG-2-interacting protein 1; SH3, Src homology domain 3; FRD, proline-rich domain; SETA, SH3 domain expressed in tumorigenic astrocytes; GST, glutathione S-transferase; ER, endoplasmic reticulum; HEK, human embryonic kidney cells; aa, amino acid(s); TBS, Tris-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Endophilins Are Main Interactors of Alix/AIP1

EXPERIMENTAL PROCEDURES

**Yeast Two Hybrid—**Alix cDNA was cloned into the yeast expression vector pGBT9 containing the GAL-4 DNA binding domain (CLONTECH) to be used as bait in a two-hybrid screen of an adult mouse brain library cDNA (CLONTECH). The screen was performed in *S. cerevisiae* Y190 according to the matchmaker two-hybrid system protocol (CLONTECH). Yeast DNA from positive clones was recovered and transformed into *E. coli* TOP10.

**SH3 Domain Deletions in SH3p4 and SH3p8—**Coding sequences for mouse SH3p4 and SH3p8 (accession numbers MMU58886 and MMU58885, respectively) were PCR-amplified and subcloned into the mammalian expression vector pCI (Promega) in-frame with the Myc epitope sequence located to the N terminus. ΔSH3p4 corresponds to SH3p4 lacking the last 37 amino acids, encompassing 60% of the SH3 domain and generated by using the unique EcoRV site. ΔSH3p8, which lacks the C-terminal fragment starting at aa 303, 4 amino acids upstream of the SH3 domain, was made using the unique SphI site of SH3p8.

**Deletion Variants of Alix—**FLAG-Alix wild type corresponds to mouse Alix cDNA (MAJ5073) subcloned into pCI FLAG. Alix-CT and -NT have been described in Missotten et al. (1). Alix-CT corresponds to a fragment spanning from amino acid 869 to 889 of Alix, whereas Alix-NT corresponds to amino acids 1–434. Both Alix cDNAs have been subcloned into pCI-FLAG vector. Alix ΔPp14 and Alix ΔPp14 ΔPp4 were made using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Briefly, two complementary primers were chosen for each deletion. The desired deletion was located within primers having 16–18 bases of correct sequences on both sides. Each primer was extended up to the temperature cycling time required for *Pfu* Turbo DNA polymerase with FLAG-Alix wild type construct as a template. Parental cDNA was then digested by the *DpnI* endonuclease, and newly synthesized deletion-containing cDNA was transformed into *XL1-Blue* supercompetent cells. Complementary primers used for ΔPp14 variants were 5′-CCAGCAGAAGACTGGTCCCTGCTGAAAACGG-3′ and 5′-CGTTGTTGAGGACCATGTTGTTGGCTGGCTTG-3′; the deletion extended from amino acids 748 to 761. The primers used to construct ΔPp4 were 5′-GCCACAGCCCTAGGATGAGCAAAAGGCCTAGC-3′ and 5′-GCTATGGGGCTTGCCATCTGAGCTTG-3′; in this case the deletion extended from amino acids 802 to 813. Alix lacking the proline-rich domain starting with Pro-717 (GGC-3) was revealed with an anti-Myc monoclonal antibody followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody. **Overlay Assays—**HEK 293 cell lysates and mouse brain extracts were prepared as described in the previous and following paragraphs, respectively. Proteins (10 μg/lane) were run on 8% SDS-polyacrylamide gels and blotted onto nylon membranes. Membranes were saturated in blocking solution (TBS, 0.1% Tween 20, 5% nonfat dry milk) for 1 h at room temperature and incubated with 10 μg/ml Myc-tagged recombinant purified protein in blocking solution. After 2.5 h, membranes were washed in TBS, 0.1% Tween 20, and the bound recombinant protein was revealed with an anti-Myc monoclonal antibody followed by a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody.

**Two-dimensional Gel Electrophoresis—**Adult mouse brain was homogenized in lysis buffer (5 mM MgCl2, 50 mM NaCl, 20 mM Hepes, pH 7.2, 0.05% Tween 20, 1 mM dithiothreitol, protease inhibitors EDTA-free). The homogenate was centrifuged at 800 × g for 20 min at 4 °C, and the supernatant was further centrifuged at 100,000 × g for 1 h at 4 °C. Cytosolic protein concentration was determined by the BCA method (Pierce).

Two hundred μg of these proteins were diluted in rehydration buffer (8 M urea, 4% CHAPS, 40 μM Tris base, 20 μM spermine, 50 μM dithiothreitol, 0.5% immobilized pH gradient (IPG) buffer) and first separated according to their isoelectric points along non-linear IPG strips, 7 cm long, pH 3–10 (Amersham Biosciences). Sample loading was performed by in-gel re-swelling. The strips were equilibrated in a solution containing 1% dithiothreitol for 15 min following by a solution containing 2.5% iodoacetamide for 10 min. The proteins were then separated according to their molecular mass using standard SDS-PAGE.

**Immunofluorescence Microscopy—**Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.2% saponin in PBS for 30 min and then incubated for 2 h with the indicated antibodies. Primary antibodies were revealed with goat anti-mouse or anti-rabbit immunoglobulin G coupled to Alexa 594 or Alexa 488 (Molecular Probes), diluted in saponin/TBS. Nuclei were stained using Hoechst 33258 (Sigma). Covergrips were mounted in Mowiol (Calbiochem) and observed with an Axiovert microscope (Zeiss) or a confocal laser-scanning microscope LSM 410 (Zeiss).

**RESULTS**

**Two-hybrid Screen for Alix-interacting Proteins—**We set out to identify and characterize Alix-interacting proteins using a yeast two-hybrid system. A cDNA encoding full-length Alix was fused to the DNA binding domain of the GAL-4 transcription factor and used to screen a mouse brain cDNA library fused to the GAL4 activation domain (the same library as that used to search for ALG-2-interacting proteins in Missotten et al. (1)). Thirty-two positive clones were obtained of 4 million transformants. Two identical clones were found to code for full-length ALG-2. One clone coded for the C-terminal half of Alix (starting at aa 456), suggesting that this domain of the protein can form oligomers.
Among 16 other clones, 8 coded for SH3p13 (4 different clones), 5 coded for SH3p4 (3 different clones), and 3 coded for SH3p8. The three proteins contain one C-terminal SH3 domain and are collectively known as endophilins since they are involved in endocytosis and are highly homologous (68% overall identity), sharing almost identical SH3 domains (16). They are also referred to as SHGL2 and endophilin I for SH3p4, which is brain-specific, SH3GL1 and endophilin II for SH3p8, which is ubiquitously expressed, SH3GL3 and endophilin III for SH3p13, which is expressed in brain and testis (12, 17). All prays obtained encompass the SH3 domain, with the shortest clone starting at aa 124 for SH3p13, and at aa 155 for SH3p4 and SH3p8. None of the other clones obtained, which will be described elsewhere, coded for other SH3 domain-containing proteins, suggesting that endophilins are specific partners for Alix in vivo.

Endophilins Interact with Alix in HEK 293 Cell Lysates and Brain Extracts—To verify that the interaction between Alix and endophilins revealed using the yeast two-hybrid system reflects their ability to interact in intact cells, we overexpressed different forms of Alix (Fig. 1A) and endophilins (SH3p4 or SH3p8) in HEK 293 cells and tested for their interactions by co-immunoprecipitations. Fig. 2 shows the results of such an experiment using cells transiently co-transfected with expression vectors coding for Myc-tagged full-length or truncated SH3p8 and for FLAG-tagged full-length or truncated Alix (Alix-NT and Alix-CT, which correspond to the N-terminal half and C-terminal half of the protein, respectively (Fig. 1A)). The overall expression of truncated and full-length Alix and SH3p8 was monitored in all cell extracts using a mixture of antibodies to Myc and to FLAG (Fig. 2c). Alix proteins were immunoprecipitated from these homogenates using an anti-FLAG monoclonal antibody, and the presence of full-length or truncated forms of Alix in the blotted immunoprecipitates was verified using a polyclonal anti-FLAG antibody (Fig. 2b). We used an anti-Myc antibody to test if Myc-tagged SH3p8 was present in the same immunoprecipitates. As seen in Fig. 2a, SH3p8 does indeed co-precipitate with full-length Alix and with Alix-CT, which contains the PRD but not with the N-terminal half of the protein (Alix-NT). Binding of SH3p8 to Alix required an intact SH3 domain, as no endophilin lacking its SH3 domain (ΔSH3p8) was detected in Alix or Alix-CT immunoprecipitates. Using a similar set of experiments we have also demonstrated that SH3p4 co-immunoprecipitates with Alix and Alix-CT (not shown). These findings strongly suggest that Alix and endophilins bind through a PRD/SH3 interaction.

Further evidence for a direct interaction between endophilins and Alix is brought by overlay experiments; lysates from

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**Fig. 1.** A, schematic representation of Alix mutants used in the study. Deletion of Alix ΔPP14 spans from aa 748 to 761, whereas that of Alix ΔPGY spans from aa 802 to 813. B, alignments between sequences containing the endophilin-binding site of mouse Alix, rat synaptojanin 1 (SJ1), and rat germinal center kinase-like kinase (GLK).
HEK 293 cells overexpressing different forms of Alix were run on SDS-PAGE and blotted onto Nylon membranes. Purified Myc-tagged recombinant SH3p4 was incubated on the membrane, and its binding was revealed with an anti-Myc antibody (Fig. 3a). SH3p4 bound strongly to a protein migrating around 100 kDa in all cell lysates (gray arrow), which was identified as endogenously expressed Alix, since it is recognized by the polyclonal anti-Alix antibody (Fig. 3c). The recombinant endophilin also bound to overexpressed full-length Alix (black arrow) and to its C-terminal half but not to its N-terminal half (not shown). The molecular mass of overexpressed Alix is higher than that of endogenous Alix due to 26 extra amino acids corresponding to two FLAG tags. Binding of endophilin to Alix occurs through an SH3/PRD interaction since (i) AlixΔPRD, which lacks the C-terminal PRD, did not bind full-length SH3p4 (Fig. 3a), and (ii) recombinant ΔSH3p4, which is truncated from its SH3 domain, did not bind any forms of Alix in the same HEK 293 cell extracts (Fig. 3c). We noticed that Alix PRD contains a 14-amino acid sequence (aa 748–761) highly homologous to the demonstrated endophilin-binding site of synaptojanin 1 (18) and of the rat germinal center kinase-like kinase (14) (Fig. 1B). This sequence of Alix represents the region of interaction with the SH3 domain of endophilin, since SH3p4 does not bind to Alix lacking these amino acids (AlixΔPP14 in Fig. 3a). Deletion of other amino acids (802–813) within the PRD of Alix had no effect on endophilin binding (AlixΔPGY in Fig. 3a).

We also used the overlay assay to test if Alix expressed in adult mouse brain binds to SH3p4. In blotted brain homogenate supernatants, SH3p4 stained 1 band at 150 kDa and 2 doublets migrating at 100 and 75 kDa (Fig. 4A). This pattern is in good agreement with that found by McPherson and coworkers (14, 19) using recombinant GST-endophilin I or endophilin I SH3 domain on brain extracts. Based on molecular masses, they identified within the endophilin-binding proteins, synaptojanin at 145 kDa and dynamin and germinal center kinase-like kinase, both migrating at 100 kDa. We show that the lower band of the 100-kDa doublet binding SH3p4 was also recognized by anti-Alix antibodies (Fig. 4A). The reason for the slightly higher molecular mass of Alix in brain compared with in HEK 293 cells is unknown. To further discriminate between 100-kDa brain proteins binding SH3p4, we performed an overlay assay on brain homogenates that had been separated on two-dimensional gels (Fig. 4B). In the 100-kDa range, SH3p4 two discrete spots with an isoelectric point in the range of the theoretical isoelectric point calculated for Alix (pI 6.15) (Fig. 4B, a). On the same membrane, the polyclonal anti-Alix antibody recognized three spots, the two more acidic co-migrating exactly with those binding SH3p4 (Fig. 4B, b). An antibody against synaptojanin (a kind gift of P. De Camilli) labeled the upper band migrating around 150 kDa (not shown). The proteins binding SH3p4, which migrate in the basic range, have not been identified.

Overexpressed Alix-CT Delineates Tubular Vesicular Structures—As described by others, we found that in HEK 293 cells, overexpressed Alix is cytoplasmic and concentrated in the perinuclear region and at the cell periphery in lamellipodia and filopodia (Fig. 5A). A similar distribution was observed with Alix-NT (Fig. 5B). In contrast, overexpressed Alix-CT containing the endophilin binding region is concentrated around perinuclear vesicles (Fig. 5, C, D, and E). These vesicles were also
seen when Alix-CT was overexpressed in 3T3 fibroblasts, NG108 neuroblastoma cells, and post-mitotic cerebellar neurones (not shown). Using confocal microscopy, we observed that some Alix-CT-induced vesicles are parts of a tubulo-vesicular network with tubes of 400–800 nm in diameter (Fig. 5D).

To test which intracellular compartments may be affected by Alix-CT, we examined the distributions of various markers in Alix-CT-transfected cells. The distribution of EEA1, an established marker for early endosomes, Lamp1, a marker for late endosomes and lysosomes, GM130, a cis-Golgi matrix protein, mtHSP70, a mitochondrial resident protein, and MitoTracker CMTMRos, a mitochondrial marker, did not significantly differ between untransfected cells and Alix-CT-transfected cells (not shown). This suggests that none of these compartments are strongly affected by Alix-CT overexpression.

In contrast, an antibody recognizing KDEL-bearing proteins, which are resident proteins of the endoplasmic reticulum lumen, stained around some of the tubulo-vesicular structures. Therefore Alix-CT may impair the shape of the endoplasmic reticulum or modify intracellular protein trafficking (Fig. 5, E and F).

Endophilins Colocalize with Alix and Enhance the Vesicular Phenotype Induced by Alix-CT—A further indication that endophilin interacts with Alix in vivo came from the perfect co-localization seen with Alix and SH3p4 overexpressed in HEK 293 cells (Fig. 6, A and B). Both overexpressed proteins co-localized in cell protrusions like lamellipodia, filopodia, and in the perinuclear region of the cytoplasm.

Knowing that the endophilin-binding site lies within Alix-CT, we performed the same type of experiments as above to verify that Alix-CT and SH3p4 interact in situ. To our surprise, co-expression of the endophilin seemed to enhance cytoplasmic vacuolization induced by Alix-CT; staining with anti-Alix antibody revealed perinuclear vacuoles considerably larger than those found in cells overexpressing Alix-CT alone (Fig. 6C). 24–48 h after transfection, 2–10 perinuclear spherical vacuoles delineated by Alix-CT were seen. In most extreme cases, vacuoles could reach a diameter of up to 6 μm (Fig. 6E). Alix-CT was almost exclusively found around these very large vacuoles, whereas SH3p4 not only co-localized with Alix-CT but was also present in the rest of the cytoplasm (Fig. 6, C and D). As in the case of Alix-CT alone, of all the intracellular markers tested, only anti-KDEL antibodies decorated the periphery of some of the very large vacuoles, suggesting that some of these structures may represent endoplasmic reticular membrane or that intracellular trafficking may be impaired (Fig. 6, F and G).

The “very large vacuole” phenotype requires an intact SH3 domain of endophilin since Alix-CT co-expressed with ΔSH3p4 not only induced the formation of tubulo-vesicular structures having the appearance of those observed in cells expressing only Alix-CT (Fig. 7C). In this paradigm, we could not detect any co-localization of Alix-CT with ΔSH3p4 (Fig. 7, C and D), dem-
showing the perinuclear localization of the vacuoles (E). They also bind to the 145-kDa marker, KDEL sequence. Some tubulo-vesicular structures delineated by Alix (arrows shown) or together with endophilins (Fig. 7, A and B). Together these data show that Alix interacts with endophilins and suggest that it modifies intracellular compartmentalization, a function synergized by endophilins.

**DISCUSSION**

By using Alix as bait in a yeast two-hybrid screen, we found that it binds to SH3 domain-containing proteins, a result that was predictable in view of the multiple potential SH3 binding motives of the long Alix PRD. More surprising to us was the specificity of the interaction, since endophilins which are identical within their SH3 domains were the only SH3-containing proteins found in our screen. However, we did not find SETA/Ruk, previously described to bind to Alix PRD (7). This could be because the SH3 domain of SETA/Ruk interacting with Alix lies within the first 151 amino acids of the 665-aa-long protein (9) and may be only rarely represented in the mouse brain library made of oligo-dT-primed inserts. Also surprising to us was the results of overlay assays suggesting that in HEK 293 cells and in brain Alix is the main endophilin interactor. Indeed, endophilins are known partners of metalloprotease disintegrins MDC5 and MDC 9 and of the β<sub>1</sub>-adrenergic receptor, which should be absent from our extracts since they are transmembrane proteins (20, 21). They also bind to the 145-kDa synaptojanin 1 and to two proteins migrating around 100 kDa, dynamin and germinal center kinase-like kinase (12, 14, 19), and to amphiphysins I and II, migrating around 120 and 80 kDa. We separated soluble brain extracts on two-dimensional gels to differentiate Alix from these latter proteins, and we found that the two more acidic, from three spots recognized by our anti-Alix antibody, bound endophilin. These Alix forms may reflect different phosphorylation states of the protein, which is a demonstrated substrate of Src in *Xenopus* (22) and contains multiple consensus phosphorylation sites for Ser/Thr kinases. Of the three forms of Alix, only the two more acidic ones bound to SH3p4, a finding that may suggest that endophilin binding depends on post-translational modifications of the protein.

The polyphosphoinositide phosphatase, synaptojanin, is involved in different steps of endocytosis, and free clathrin-coated vesicles accumulate in living lamprey synapses microinjected with a peptide blocking the SH3 domain of endophilin (18). This peptide, PP19, lying within the synaptojanin PRD is homologous to the endophilin-binding site of germinal center kinase-like kinase (14) and MDC9 (20) and also to 14 amino acids of Alix PRD (aa 748–761) (Fig. 1B). This Alix peptide contains the endophilin-binding site with a consensus PXRP-PPP conserved in *Xenopus* and in *Caenorhabditis elegans*, reminiscent of the endophilin III binding motif PXRPXPXR that Cesareni and co-workers (23) define using recombinant peptides.

**Interaction between Alix-CT and Endophilin Deforms Intracellular Membranes**—In our case, neither overexpression of Alix nor of Alix-CT, which both contain the endophilin-interacting site, had any detectable effect on dextran- or clathrin-mediated transferrin endocytosis (not shown). This lack of effect may suggest that Alix could interact and regulate endophilins not at the plasma membrane but in cytoplasmic

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**FIG. 5. Cellular localization of overexpressed full-length and mutated forms of Alix.** In HEK 293 cells, overexpressed Alix, Alix-NT, and Alix-CT proteins were revealed by immunofluorescence using a polyclonal anti-Alix antibody (A–E). Bar, 12 μm in D and 10 μm in all other fields. Overexpressed Alix (A) and Alix-NT (B) both localize in the cytoplasm and are particularly enriched at cell edges and protrusions. In contrast, Alix-CT induces and delineates tubulo-vesicular structures concentrated within the perinuclear region (C). Confocal analysis demonstrate the tubulo-vesicular nature of the compartment induced and delineated by Alix-CT. The bottom part shows a z section (height: 9.1 μm) along the white line (D). Tubulo-vesicular structures induced by overexpression of Alix-CT (E) may affect the ER. Cells were double-labeled for Alix and for the ER marker, KDEL sequence. Some tubulo-vesicular structures delineated by Alix (arrows in E) were also stained by ER marker (arrows in F). DNA labeled with Hoechst shows the perinuclear localization of the vacuoles (E). N, nucleus.
Fig. 6. Alix and endophilin co-expression in HEK 293. HEK 293 cells were co-transfected with Alix and Myc-tagged SH3p4 (A and B) or Alix-CT and Myc-tagged SH3p4 (C–G). Transfected cells were immunostained with polyclonal anti-Alix (A, C, E, and F) and co-immunostained with anti-Myc monoclonal antibodies to localize endophilins (B, D, and E) or with anti-KDEL antibody that stains ER resident proteins (G). In E, cells were observed with a confocal microscope. Bar, 10 μm. In co-transfected HEK 293 cells, Alix (A) perfectly co-localizes with SH3p4 (B). Endophilin co-expression dramatically enhances the vacuolization effect of Alix-CT; co-expression of the Alix-CT with full-length SH3p4 leads to the appearance of large vacuoles around which most of Alix-CT (C) and some of the endophilin (D) concentrate. In confocal analysis (E) Alix-CT (red) delineates a few perfectly spherical vacuoles; SH3p4 (green), even though concentrated at the periphery of the vacuoles, is also found in the rest of the cytoplasm. The bottom part of E shows a z section (height: 19 μm) along the white line. N, nucleus. Vacuolization induced by overexpression of Alix-CT and of SH3p4 may affect the endoplasmic reticulum, as some perinuclear vacuoles delineated by Alix (arrows in F) were also stained by ER marker (arrows in G). Arrowheads point to vacuoles, which are only Alix-positive. DNA was labeled with Hoechst.

Fig. 7. A–D, an intact PRD in Alix-CT and an intact SH3 domain in SH3p4 are required to induce cytoplasmic vacuolization. HEK 293 cells were co-transfected with FLAG-tagged Alix-CT ΔPRD and full-length Myc-tagged SH3p4 (A and B) or with FLAG-tagged Alix-CT and Myc-tagged ΔSH3p4 (C and D). The cells were stained with polyclonal anti-Alix (A and C) together with monoclonal anti-Myc to reveal endophilin (B and D). PRD deleted from Alix-CT does not induce cytoplasmic vacuoles (A and B), and endophilin lacking its SH3 domain (ΔSH3p4) does not enhance Alix-CT induced vacuolization; in this case the tubulo-vesicular structures have the size and appearance of those seen with Alix-CT alone (C and D). E–H, Alix-CT-induced vacuoles are insoluble in Triton X-100. HEK 293 cells were transfected with FLAG-tagged Alix-CT alone (E and F) or together with Myc-tagged SH3p4 (G and H). Cells were stained with polyclonal anti-Alix (E and G) together with monoclonal anti-Myc to reveal endophilin in H; the staining was performed after a pretreatment with 1% Triton X-100 for 3 min before paraformaldehyde fixation. F is a phase contrast micrograph of the same field as that seen in E. Note in H that residual SH3p4 is found exclusively around vacuoles co-localizing with Alix-CT. Bar, 10 μm in all fields.
compartments. Recently, Farsad et al. (24) demonstrate that endophilin B, a protein with homology to endophilin 1, is localized to the Golgi complex, thereby underscoring a potential role of endophilin family members in diverse tubulo-vesicular membrane-trafficking events in the cell. This hypothesis is in good agreement with our finding that overexpression of Alix-CT leads to accumulation of perinuclear tubulo-vesicular structures, which were transformed into a few very large vacuoles upon co-expression with endophilins. Overexpressing endophilins alone had no vacuolization effect, and endophilin lacking the SH3 domain did not synergize with Alix-CT. Furthermore, Alix-CT devoid of its PRD region and, therefore, unable to interact with endophilins had no vacuolating effect in HEK 293 cells. Our current hypothesis is that Alix-CT deforms intracellular membranes by interacting with and modifying the activity of endogenously expressed endophilin and that increasing cellular membranes by interacting with and modifying the activity of endophilins had no vacuolization effect in HEK 293 cells.

It has been difficult to determine precisely which intracellular compartment Alix-CT and endophilins do affect. Proteomic studies demonstrate that Alix is highly enriched in phagosomes (25) and in exosomes, these latter originating from multi-vesicular bodies that are intermediates between early and late endosomes (26). In our hands, neither early nor late endosomes were drastically affected by Alix-CT and endophilin overexpression. A marker of ER resident proteins (KDEL) was distributed around some Alix-CT/endophilin-induced tubulo-vesicular structures and vacuoles, suggesting that Alix-CT overexpression leads to swelling of parts of the ER or impairs trafficking of some ER proteins. Interestingly, the Alix-CT/endophilin-delineated structures induced by the overexpression were resistant to Triton-X100. Because we have not found any abnormal cholesterol concentration in Alix-CT-stained compartments (not shown), we favor the hypothesis that the resistance to detergent is due to Alix-CT and endophilins being caught in a mesh of cytoskeletal proteins stabilizing the tubules and vacuoles.

Protective Effect of Alix-CT in Cell Death—Overexpression of the C-terminal half of Alix/AIP1 was shown by Vito et al. (4) to impair apoptosis after serum deprivation, and we have recently observed a similar death-blocking effect of overexpressed Alix-CT in cerebellar post-mitotic neurons.2 One mechanism put forward by Vito et al. (4) to explain this protective effect was that Alix-CT may sequester ALG-2, which is necessary for death to occur. However, we have not seen colocalization of ALG-2 with Alix-CT (not shown). Our demonstration that Alix-CT perturbs intracellular compartmentalization, possibly by binding to endophilins, begs the question of how localization and activity of the known actors of the death program may be affected by this vacuolization.

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