Cloning of AIP1, a Novel Protein That Associates with the Apoptosis-linked Gene ALG-2 in a Ca\(^{2+}\)-dependent Reaction*

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ALG-2 is a 22-kDa calcium-binding protein necessary for cell death induced by different stimuli in 3DO T-cell hybridoma. 3DO cell clones depleted of ALG-2 protein exhibit normal caspases activation, suggesting that ALG-2 function is required downstream or is independent of caspase proteases activity for apoptosis to occur. Using the yeast two-hybrid screening system, we have isolated and characterized the mouse cDNA encoding for ALG-2 interacting protein 1 (AIP1), a novel protein that interacts with ALG-2. ALG-2 and AIP1 colocalize in the cytosol and the presence of calcium is an indispensable requisite for their association. Sequence alignment shows that AIP1 is highly similar to BRO1, a yeast protein related to components of the Pkc1p-MAP kinase cascade.

Overexpression of a truncated form of AIP1 protects two different cell types from death induced by trophic factors withdrawal; thus, our data indicate that AIP1 cooperates with ALG-2 in executing the calcium-dependent requirements along the cell death pathway.

Programmed cell death (PCD) is a physiologically regulated cell type-specific deletion that takes place during various developmental stages of multicellular organisms and is essential for the establishment and the maintenance of cellular homeostasis (1). PCD occurs by apoptosis, which refers to the morphological changes that can be observed in cells undergoing PCD. These include plasma membrane blebbing, cell shrinkage, chromatin condensation, and DNA degradation (2). The process eventually culminates with the fragmentation of the cells into apoptotic bodies that, in vivo, are rapidly phagocytosed by the surrounding cells (3).

The suicide signal can be received and transduced into the cell through death receptors that initiate a signaling cascade, which shortly leads to cell demise (4). A number of molecules have been identified that either regulate the death pathway or are able to modulate the death signal. Most attention has recently focused on the interleukin-1β converting enzyme-like proteases, a family of at least 10 related mammalian cysteine proteases collectively named caspases (5). These proteins are considered the executioners of mammalian apoptosis by virtue of two seminal observations: (a) ectopic overexpression of interleukin-1β converting enzyme, as well as other members of the family, results in protease activation and induces apoptosis (6, 7), and (b) specific inhibitors of these proteases inhibit cell death (8, 9). Accordingly, the caspase paradigm asserts that cells undergoing apoptosis accomplish their suicide program by activating a hierarchy of caspases. However activation of caspases does not always correlate with induction of apoptosis (10, 11), and in some circumstances, caspase activity is required for protection from apoptosis (12).

In addition to proteases activation, numerous evidence demonstrates that alterations in intracellular calcium play an important role during apoptosis. For some time it has been proposed that DNA fragmentation, a general hallmark of apoptosis, is a Ca\(^{2+}\)-dependent event that requires a Ca\(^{2+}\)-activated endonuclease (13, 14). Induction of apoptosis triggers an increase of [Ca\(^{2+}\)], as well (15, 16), and intracellular chelators of Ca\(^{2+}\) inhibit cell death (17). As consequence of Fas stimulation, calcium is immediately mobilized from intracellular stores (17), and lymphoid cells rendered deficient for the calcium release channels are resistant to apoptosis induced by different stimuli (18, 19). Of interest, and consistent with the literature, the antiapoptotic protein Bcl-2 decreases calcium release from the endoplasmic reticulum stores (20). Considering these data altogether, the existence of a Ca\(^{2+}\)-sensitive step(s) along the cell death pathway is clearly apparent. Although a precise picture of how Ca\(^{2+}\) exerts these effects at the molecular level is still not available, it is likely that transduction of Ca\(^{2+}\)-regulated signals occurs through Ca\(^{2+}\)-binding proteins.

Members of the EF hand Ca\(^{2+}\)-binding protein family exhibit Ca\(^{2+}\) affinities and binding kinetics compatible with concentration and time range of Ca\(^{2+}\) wave (21). The functional and structural Cu\(^{2+}\)-binding unit is the helix-loop-helix motif commonly called EF hand domain. One of them, ALG-2, is a 22-kDa protein that was identified during a screening for genes involved in apoptosis (22, 23). T-cell hybridoma clones depleted of ALG-2 via transfection of the antisense cDNA are resistant to cell death induced by diverse stimuli, including T-cell receptor and Fas stimulation as well as dexamethasone, staurosporine, and ceramide treatment. Nonetheless, in these ALG-2-deficient clones caspases are normally activated upon induction of cell death, as determined by cleavage of poly(ADP-ribose)polymerase and of a fluorescent substrate (24).

In this paper we report the cloning of AIP1, a novel gene that interacts with ALG-2 in a calcium-regulated fashion. Overexpression of a deletion mutant of AIP1 protects HeLa and COS cells from apoptosis induced by serum starvation; thus AIP1 might mediate, at least in part, the ALG-2 requirement for apoptosis.
Cloning of AIP1

| DNA-binding          | Activating     | β-galactosidase filter assay |
|----------------------|----------------|-----------------------------|
| pLAM5                | pGAD10-TH28    | White                       |
| pAS2-ALG2FL          | pGAD10-TH28    | White                       |
| pAS2-ALG2AN          | pGAD10-TH28    | White                       |
| pAS2-ALG2ΔC          | pGAD10-TH28    | White                       |

**a**, β-galactosidase activity was determined by a filter assay for yeast transformed with the indicated plasmid as described under “Experimental Procedures”; b, schematic representation of the full-length ALG-2 and the deletion constructs used in the assay. The black boxes represent the calcium binding domains; the numbers indicate the amino acidic position.

### EXPERIMENTAL PROCEDURES

**Two-hybrid Screen and β-Galactosidase Assays**—The two-hybrid screening was conducted using the Matchmaker system from CLONTECH according to the manufacturer’s instructions. Yeast strain Y190 was transformed with the corresponding bait plasmids by lithium acetate/polyethylene glycol 4000 procedure and selected on synthetic dropout plates lacking tryptophan. Selected colonies were then analyzed for expression of the GAL4-bait fusion protein by immunoblot analysis.

For library screening, yeast strain Y 190 expressing GAL4-ALG-2 fused protein was sequentilly transformed with a mouse liver cDNA library cloned in the pGAD10 vector (CLONTECH). 2 × 10⁶ clones were analyzed. Transformed yeast were selected on synthetic dropout/agar plates lacking leucine, tryptophan, and histidine in the presence of 50 mM KCl, 1 mM MgSO₄, 37.5 mM 3-aminotriazol (Sigma) and grown for 5 days at 30 °C. Colonies positive for growth on selective media were blotted on filter paper (Whatman No. 5), permeabilized in nitrogen liquid, and placed on another filter soaked in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 37.5 mM β-mercaptoethanol) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Colonies that developed color were re-streaked on selective plates to allow plasmid segregation of the other plasmid constructs used were made by polymerase chain reaction performed with primers containing appropriate restriction sites or epitope tags as needed. GST fusion proteins were made in pGEX vectors (Amersham Pharmacia Biotech). Plasmid constructs were confirmed by partial sequencing and immunoblot analysis.

**Cell Culture and Antibodies**—293T, L66, HeLa, and COS cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. 3DO cells were cultured in RPMI, 5% fetal calf serum. The polypeptide DEIKKERESLENLIK was used to generate rabbit polyclonal antisera. The whole rabbit serum was affinity-purified on CNBr-activated Sepharose beads (Amersham Pharmacia Biotech) coupled with the antigenic peptide.

HeLa and COS cells were transfected with lipofectAMINE (Life Technologies, Inc.) following the manufacturer’s indications. 293T cells were transfected by the calcium phosphate method.

**Immunoblot Analysis and Coprecipitation**—Cell lysates were made in RIPA buffer, and protein concentration was determined by Bio-Rad protein assay; 10–20 μg of protein was subjected to polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane and incubated with primary antibody followed by a secondary antibody horseradish peroxidase-conjugated (Promega). Blots were developed using SuperSignal (Pierce) and visualized by exposure to autoradiography film. For coimmunoprecipitation experiments, cells were lyzed in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and a protease inhibitor mixture). Lysates were divided and immunoprecipitated with the indicated antibodies; immunocomplexes were bound to protein A-agarose beads and resolved by SDS-PAGE as described above.

**Immunofluorescence and Subcellular Fractionation**—L66 cells were grown in chamber slides, fixed in 4% paraformaldehyde for 15 min at room temperature, and then permeabilized in phosphate-buffered saline, 0.5% saponin. AIP1 was visualized by incubating the cells with the affinity-purified anti-AIP1 antiserum for 30 min, washing several times with phosphate-buffered saline, 0.5% saponin, and then incubating for 30 min with a fluorescein isothiocyanate-conjugated mouse
anti-rabbit. L66 cells hybridized in the presence of an excess (10 μg) of immunogenic peptide served as negative control. All steps were done at room temperature; the slides were analyzed with a confocal laser scanning microscope.

For subcellular fractionation, cells were homogenized with a glass Pyrex homogenizer in lysis buffer: 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, and a mixture of protease inhibitors. Nuclei were removed by centrifuging the homogenates at 1,000 × g for 10 min, and the supernatant was centrifuged at 130,000 × g for 60 min to obtain the cytosolic and the membrane fraction.

In Vitro Binding—Immobilized GST-ALG-2 fusion protein (1 μg) was washed three times in wash buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) and added to 500 μl of binding buffer, which consisted of wash buffer supplemented with 1 mM EGTA and either CaCl2 or MgCl2. The amount of each of these reagents needed to obtain a given concentration of free cation was calculated with Free Calcium program (25).

cDNAs encoding FLAG-tagAIP1 and ALG-2 proteins were in vitro transcribed/translated as described above. The lysate (5 μl) was added to the immobilized GST-ALG-2 protein resuspend in binding buffer and incubated for 1 h at room temperature with gentle rotation. In the experiment shown in Fig. 4b, right panel, 5 μl of in vitro synthesized FLAG-AIP1 and ALG-2 proteins were incubated in binding buffer containing the indicated cation and were immunoprecipitated with an anti-FLAG antibody (Kodak) and protein AG-Sepharose beads (Pierce).

The beads were then washed six times in binding buffer, resuspend in loading buffer, and the sample analyzed by SDS-PAGE followed by autoradiography.

Cell Death Assay—COS and HeLa cells were transfected with 2 μg of pcDNA3 expressing the indicated cDNA together with 0.2 μg of pCMV-β-gal (CLONTECH). 24 h after transfection the cells were treated with 1 μM etoposide or 1 μM staurosporine (Sigma) for 7 h or starved lowering the fetal calf serum concentration to 0.1%. β-Galactosidase activity was visualized by fixing the cells in 0.2% glutaraldehyde for 10 min followed by staining in phosphate-buffered saline containing 20 mM each K3Fe(CN)6 and K4Fe(CN)6-H2O and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside for 1–3 h at 37 °C. The number of live blue cells and blue cells with apoptotic morphology were counted in at least four fields.

RESULTS

Two-hybrid Screening—To identify ALG-2 interacting proteins, we performed a two-hybrid screen with the full-length ALG-2 cDNA fused to the GAL4 DNA binding domain (GAL4BD). A plasmid library of fusion between the GAL4 transcription activation domain (GAL4AD) and cDNAs from mouse liver was screened for interaction with GAL4BD-ALG-2 fusion protein in the yeast reporter strain Y190. A total of 14 clones were isolated that activated the β-galactosidase reporter when approximately 2 × 106 plasmids were transformed. Restriction mapping and sequencing of these 14 cDNAs revealed that three clones (TH 2, TH 28, and TH 37) had the same insert containing an open reading frame coding for 402 amino acids fused to GAL4AD. Further assays were then performed with clone TH 28 in the reporter yeast strain. As summarized in Table I, this library clone did not activate β-galactosidase by itself or when coexpressed with the empty GAL4BD vector or with a control plasmid. Conversely, it strongly interacted with GAL4BD-ALG-2, and no association was reported with truncated forms of ALG-2 deleted at the amino or carboxyl
terminus. Hence, only the full-length ALG-2 specifically interacts with TH 28 in yeast.

**AIP1 Cloning and Expression**—On Northern blots clone TH 28 detects a ~4-kb transcript ubiquitously expressed, and an additional, less abundant, 7-kb transcript (Fig. 1a). The 3DO T-cell hybridoma expresses only the 4-kb mRNA, and the levels of transcription are not regulated by T-cell receptor stimulation or glucocorticoid treatment (data not shown).

Using clone TH 28 as probe, we screened a mouse liver cDNA phage library and cloned the full-length cDNA, that we named AIP1 for ALG-2 Interacting Protein 1. The nucleotide sequences of several phage clones indicated that AIP1 consists of 840 amino acids (Fig. 1b). Accordingly, the in vitro transcription/translation of full-length AIP1 cDNA generates a single polypeptide of ~105 kDa (Fig. 1c). A BLAST search revealed that several homologues of A21P1 have been cloned: YNK1 in the nematode *Caenorhabditis elegans* (26), palA in the filamentous fungus *Aspergillus nidulans* (27), and BRO1 in the yeast *Saccharomyces cerevisiae* (28). Functional and genetic evidence suggests that Bro-1 and palA participate in signal transduction pathways.

**AIP1 and ALG-2 Interact in Vivo**—A rabbit antiserum was raised against the polypeptide Asp^{561}-Leu^{774} of the AIP1 amino acid sequence, which is also included in the TH 28 sequence. Immunoblot analysis performed on cell lysates from L66 fibroblasts and 3DO T-cell hybridoma indicates that this antiserum recognizes the endogenous AIP1 protein as a single band migrating with an apparent molecular mass of 105 kDa. 293T cells transfected with AIP1 cDNA exhibit a specific band that comigrates with the endogenous AIP1 (Fig. 2a). Both signals from murine cell lysates and transfected 293T cells were specific, because the immunoreactivity was abolished by competition with the immunogenic peptide (data not shown).

We then tested whether the interaction between ALG-2 and AIP1 also occurs in mammalian cells. As shown in Fig. 2b, HA-flagged AIP1 specifically coprecipitates with ALG-2 in lysates from 293T-transfected cells. As expected, HA-flagged TH 28 coprecipitates with ALG-2 as well. This latter observation suggests that in overexpression experiments TH 28 can potentially inhibit the association between ALG-2 and AIP1.

In addition, in lysates from untransfected L66 cells and the T-cell hybridoma, the endogenous AIP1 coprecipitates with the endogenous ALG-2 (Fig. 2c). Thus, ALG-2 and AIP1 physically interact in mammalian cells.
ALG-2 and AIP1 Colocalize in the Cytosol—To determine the cellular localization of AIP1, L66 fibroblasts were permeabilized and stained with the anti-AIP1 antibody followed by fluorescein isothiocyanate-conjugated mouse anti-rabbit (A) and in the presence of 10 μg of immunogenic peptide (B). C, subcellular fractionation of lysates from L66 cells. 10 μg of proteins from unfractionated lysate or from cytosolic and membrane fraction were resolved by SDS-PAGE and immunoblotted using the anti-AIP1 or the αALG-2 antiserum.

**Fig. 3. Cellular localization of AIP1.** Confocal images of L66 fibroblast stained with anti-AIP1 antibody followed by fluorescein isothiocyanate-conjugated mouse anti-rabbit (A) and in the presence of 10 μg of immunogenic peptide (B). C, subcellular fractionation of lysates from L66 cells. 10 μg of proteins from unfractionated lysate or from cytosolic and membrane fraction were resolved by SDS-PAGE and immunoblotted using the anti-AIP1 or the αALG-2 antiserum.

ALG-2 and AIP1 Colocalize in the Cytosol—To determine the cellular localization of AIP1, L66 fibroblasts were permeabilized and stained with the anti-AIP1 antiserum. Fluorescence microscopy revealed that AIP1 has a granular cytoplasmic distribution, consistent with a cytosolic localization of the protein (Fig. 3). Subcellular fractionation experiments confirmed this observation. Immunoblot analysis of fractionated lysates from L66 cells showed that AIP1 was present mostly in the cytosolic fraction, although a portion of the protein also distributes in the membrane-containing fraction (Fig. 3C). In the same assay ALG-2 localizes essentially in the cytosolic fraction. Together with the microscopic results, these data implicate that ALG-2 and AIP1 colocalize in the cytosolic compartment.

The Binding of AIP1 to ALG-2 Is Calcium-dependent—Intracellular free Ca2+ modulates the activity of Ca2+-binding proteins by binding to their EF hand domain(s). Some of these proteins undergo major conformational reorganization upon Ca2+ binding, which regulates the interactions with target protein(s) (21). Since ALG-2 is known to undergo such a Ca2+-dependent conformational change (22), we asked whether Ca2+ could modulate the interaction with AIP1. In these experiments we used several [Ca2+]i, ranging from the physiological amount of free Ca2+ present in resting cells (~50 nM) to a concentration that is reached during Ca2+ flux from storage compartments (1–2 μM). As shown in Fig. 4a, in vitro transcribed/translated AIP1 and GST-ALG-2 fusion protein interact only when at least 750 nM free Ca2+ is present in the binding buffer. The specificity of Ca2+ was investigated by
replacing Ca$^{2+}$ with another divalent cation, Mg$^{2+}$. At physiological intracellular concentrations of free Mg$^{2+}$ (900 mM), ALG-2/AIP1 complex did not occur (Fig. 4b). Overall, these data indicate that ALG-2 and AIP1 interact in a Ca$^{2+}$-specific manner and only when intracellular concentrations of free Ca$^{2+}$ increase above the normal resting level.

**DISCUSSION**

Work from many laboratories has implicated Ca$^{2+}$ signaling in the regulation of apoptosis in mammalian cells. In lymphoid cells, antigen-receptor engagement results in a sustained increase in intracellular [Ca$^{2+}$], followed by alternative responses such as apoptosis or cellular activation and proliferation. Calcium ionophores induce apoptotic cell death in a variety of experimental systems, suggesting that an increase in intracellular [Ca$^{2+}$] is sufficient to signal the cell to enter the apoptotic program. It appears, therefore, that while Ca$^{2+}$ is essential for a response to occur, the type of response is more dependent on the activation of specific pathways. Yet, the critical target(s) relating this calcium flux to cellular apoptosis are only partially identified. ALG-2 is a Ca$^{2+}$-binding protein shown to be directly involved in the control of programmed cell death; however, knowledge concerning the biochemical mechanisms involving ALG-2 is still incomplete. In this study we have identified and characterized AIP1, a protein that interacts with ALG-2 in a Ca$^{2+}$-dependent manner. The presence of Ca$^{2+}$ is essential for this binding, presumably due to conformational requirements necessary for association which are satisfied only when ALG-2 is in a Ca$^{2+}$-loaded state. The range of [Ca$^{2+}$] needed for the association of the two proteins in vitro is compatible with physiological levels of cytosolic Ca$^{2+}$. In fact, while in resting cells Ca$^{2+}$ is maintained at relatively low concentration (10–100 nM), extracellular flux or release from intracellular stores can result in an increase to the 500–1000 nM range required for the interaction. This observation suggests that AIP1 and ALG-2 are dissociated in resting cells and associate following stimulation that results in intracellular [Ca$^{2+}$] rise.

Expression of a truncated form of AIP1 partially inhibits apoptosis evoked by some stimuli. The simplest interpretation of these experiments is that interaction between ALG-2 and AIP1 is required for cell death, and the polypeptide generated by TH 28 acts as a negative inhibitor of AIP1 by competing for binding to ALG-2. However, it is possible that the truncated protein encoded by TH 28 might still perform some of the functions executed by AIP1. This would explain why the inhibition of apoptosis that we observed was only partial and evident with only a few stimuli. More specific reagents will allow us to precisely determine the role of this association in programmed cell death and in cell physiology in general.
AIP1 shows a high degree of homology to BRO1, a *S. cerevisiae* protein of 844 amino acids that is 22% identical to AIP1 over the entire sequence (28). Genetic and functional evidence relates BRO1 to components of the Pkc1p-MAP kinase cascade. BRO1 mutants, in fact, display phenotypes similar to those caused by deletion of *BCK1*, a gene encoding a MAP/ERK

**Fig. 5. Overexpression of TH 28 partially protects from apoptosis.** a, HeLa and COS cells were transfected with the indicated plasmids together with pCMV-β-gal and induced in apoptosis by serum starvation for 16 h or treated with 1 μM etoposide (b) or 1 μM staurosporine for 7 h (c). After treatment cells were stained and examined by phase contrast microscopy. Numbers are the percentage of apoptotic blue cells over the total blue cells. Data in b and c represent mean ± S.D. of three experiments. Data in a are representative of two experiments done in duplicate.
kinase that mediates maintenance of cell integrity. BRO1 mutations result in a temperature-sensitive osmoremedial growth defect, which is suppressed by Ca\(^{2+}\). Since BRO1 itself lacks calcium binding domains, it is likely that, in yeast, the function of BRO1 is regulated by an ALG-2-like protein.

The homology between AIP1 and BRO1 suggests that one possible mechanism by which the interaction ALG-2/AIP1 might control programmed cell death is by activation of signal transduction pathways linked to MAP kinases. Consistent with this idea is the evidence that p38 MAP kinase is required for apoptosis induced by trophic factors withdrawal (29). However, further studies are needed to define whether Ca\(^{2+}\)-regulated signals influence, through AIP1, activation of MAP kinases.

Involvement of other Ca\(^{2+}\)-dependent protein kinases and phosphatases in apoptosis has been demonstrated previously. Expression of calcineurin, a Ca\(^{2+}\)-dependent protein phosphatase that functions in T-cell activation, rapidly induces apoptosis in the absence of growth factors (30). Because calcineurin activates the transcription factor NF-AT, it is possible that an alteration in gene transcription influences the decision to enter apoptosis. This is also supported by the recent evidence that sequestering of active calcineurin (31). Whether AIP1 plays a role in this scenario remains to be determined.

\(\text{palA}\) is the \(A. \text{nidulans}\) gene encoding for a \(798\)-residues protein which displays \(25.6\%\) similarity to AIP1 and participates in the regulation of pH-dependent gene expression (27). A homologue of AIP1 also exists in \(C. \text{elegans}\), namely \(YNK1\) (26), which encodes for a \(798\)-amino acid protein. Interestingly, YNK1, \(\text{palA}\), and AIP1 all conserve, near their carboxyl terminus, a proline-rich region containing the PXXP consensus sequence of SH3 domain-binding motifs. It is, therefore, likely that AIP1 is involved in a variety of complex cellular responses, of which apoptosis is only one of the possible outcomes.

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