Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein

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Abstract

ALG-2 is a EF hand calcium binding protein with sequence homologies to calmodulin. Vito *et al* have shown that ALG-2 expression is required for apoptosis following a number of death stimuli,¹ although nothing is known about the effectors which underlie ALG-2 function. Here we have used ALG-2 as bait in a yeast two hybrid screen of a mouse brain cDNA library. We found that ALG-2 binds to itself and to a novel protein that we call ALG-2 interacting protein X, Alix. Using co-immunoprecipitation experiments, we confirmed ALG-2/ALG-2 binding and demonstrated that this interaction is calcium independent. ALG-2/Alix interaction was also validated by co-immunoprecipitation, but in this case, the binding was found to be strictly calcium dependent. Alix seems highly conserved throughout evolution since it shows significant homologies to a putative *C. elegans* protein (YNK-1) and to proteins of *A. nidulans* (PalA) and *S. cerevisiae* (BRO1). Alix is a potential regulator or downstream effector of ALG-2 action.

Keywords: cell death; calcium; ALG-2; Alix; Bro1; PalA

Abbreviations: ALG-2; apoptosis-linked-gene-2; Alix; Alg-2 interacting protein X

Introduction

The cell death program underlying apoptosis is a highly regulated process, controlled by members of the Bcl-2- and caspase-gene families with cell fate resulting from a balance of death-inducing and survival-promoting signals. Cytosolic calcium is one such signal which, depending on the cellular context, is able to promote either cell death or survival. Hence, in some cases increased Ca^{2+} concentration, such as following thapsigargin- or Ca^{2+} ionophore-treatment, is capable of inducing apoptosis. Furthermore, sustained calcium release following TCR stimulation in thymocytes or

NMDA receptor activation in neurons, is essential for the death process. In contrast, elevation in intracellular Ca^{2+} can also rescue cells from certain death stimuli. For example chronic depolarisation which induces Ca^{2+} entry in neurons can protect these cells from neurotrophic factor deprivation and hematopoietic cells are protected from IL-3 deprivation by Ca^{2+} ionophores.² The molecular mechanisms which differentially drive an elevation in intracellular Ca^{2+} into cell death or survival are poorly understood.

Recently Vito *et al* have characterised the Apoptosis Linked Gene-2 (ALG-2)^{1,3} which codes for a Ca^{2+} binding protein. Inhibition of ALG-2 expression protected lymphoid cells against TCR-induced cell death although it did not appear to interfere with other aspects of TCR signalling as neither IL-2 production nor Fas induction were impaired in cells lacking ALG-2. These cells were also protected against Fas-, ceramide- (a downstream effector of Fas) and dexamethasone-induced death. The lack of ALG-2 expression blocked cell death without impairing caspase activation suggesting that ALG-2 exerts its function at a distal step common to several apoptotic pathways.⁴ ALG-2 encodes a protein of 191 amino acids containing five potential calcium binding sites with strong homology to calmodulin 'EF hands' motifs.⁵ It also shows high level of homology to sorcin a protein tightly associated with the ryanodine receptor,⁶ and to grancalcin a cytosolic protein identified in neutrophils and monocytes which displays Ca^{2+} dependent translocation to membranes.⁷

Because of its homology with calmodulin, which after binding to calcium, interacts with and modifies the properties of a number of proteins including enzymes,⁸ we thought that ALG-2 may interact and activate downstream effectors of the apoptotic program in a calcium regulated way. As a first step to establish the nature of such effectors, we used the yeast two hybrid system to characterise ALG-2 interacting proteins. An adult mouse brain library was screened and several clones identified. One of these clones encoded part of ALG-2. Another clone encoded a novel mouse protein that we call Alix (ALG-2 interacting protein X). Alix shows strong homologies to YNK-1 an open reading frame in *C. elegans* and to proteins in *S. cerevisiae* and *A. nidulans*. We found that the interaction between ALG-2 and Alix is strictly dependent on calcium, suggesting that Alix could participate in a regulatory Ca^{2+} dependent pathway controlled by the ALG-2 protein.

Results and Discussion

Two-hybrid screen for ALG-2 interacting proteins

We set out to identify and characterise ALG-2 interacting proteins using the yeast two-hybrid system. A cDNA encoding

the full-length ALG-2 was fused to the DNA binding domain of the GAL4 transcription factor and used to screen a mouse brain cDNA library fused to the GAL4 activation domain. Fourteen positive clones were obtained from approximately 1 million transformants. Eleven of these cDNAs were identical and contained inserts of 700 bp. These clones are currently being characterised and will not be described herein. Among the three remaining cDNA clones, one was a partial 900 bp insert encoding the last 92 amino acids of the ALG-2 C-terminal region. This suggested that ALG-2 interacts with itself and that the C terminal half of the protein is sufficient for binding. The two remaining clones analyzed were identical cDNAs of 1700 bp, containing an unknown open reading frame encoding 401 amino acids followed by a stop codon and approximately 500 bp of untranslated sequence. We next used this cDNA to screen a λ gt10 mouse cDNA library to obtain a full-length clone. This allowed us to isolate a 1710 bp fragment which although containing a large overlap (500 bp) with the 5' end of the two hybrid screen product did not include a translation initiation codon. This partial cDNA was used to scan EST databases. A mouse EST clone was found that contained sequences overlapping with the 1710 bp cDNA and a putative ATG initiator codon, fitting a Kozak consensus sequence⁹ (accession number: GenBank W97887). The 5' end of the complete cDNA was cloned by RT-PCR, using mouse brain RNA as template. The three partial cDNA fragments were then ligated using overlapping sequences and appropriate restriction enzymes to generate a full length cDNA. The protein encoded by this cDNA was named Alix, for ALG-2 interacting protein X.

Subsequent sequence analysis of the complete EST clone revealed an open reading-frame of 222 amino acids. The nucleotide sequences encoding the first 158 amino acids as well as the last 64 amino acids of the EST clone were identical to the 5' end and the 3' end of Alix cDNA, respectively. Since the large central part of the full-length Alix cDNA was missing, this EST clone might represent an alternative spliced variant of Alix. We called this second clone Alix SF (Alix short form).

Mouse Alix shows strong homologies with proteins in *C. elegans*, *A. nidulans*, *S. cerevisiae* and with mouse Rhoophilin

The deduced amino acid sequence from the mouse Alix cDNA is shown in Figure 1a. It encodes a protein of 869 amino acids with a predicted molecular weight of 96 kD. The last 140 amino acids of the protein are particularly rich in proline and tyrosine and contain several Src homology domain 3 (SH3) binding motives (PXXP, boxed in grey) and WW binding domains (XPPXY, aa 723–727 and 834–838). Since ALG-2 does not contain sequences reminiscent of neither SH3 nor WW domains,¹⁰ this suggests that the WW- and SH3-binding domains of Alix are involved in interactions with proteins other than ALG-2. An additional feature of the protein is the presence of a consensus tyrosine phosphorylation site (black box, consensus: Prosite: PDOC00007: [RK]-X(2,3)-[DE]-X(2,3)-Y).

Comparison of the predicted amino acid sequence with sequences in Genbank showed that Alix is homologous

(40.4% identity) to YNK-1, the putative 98 kD product of an open reading frame in *C. elegans* (Genbank accession number: Z29561). In addition, Alix shows significant homologies with YOR 275c (28.6%, identity), a 661 amino acids-open reading frame from *S. cerevisiae* (accession number Q12033), and to known proteins in *A. nidulans* (PaIA, 798 aa, 27.4% identity) and in *S. cerevisiae* (BRO1, 844 aa, 24.5% identity)^{11,12} (Figure 1b). Noteworthy is the perfect conservation of the putative Tyr phosphorylation site between all proteins. Despite these homologies, conservation between the different proteins is lower within their COOH terminal region. In particular, the open reading frame YOR 275c encodes a protein lacking the proline rich region found in Alix, YNK-1, PaIA and BRO1. In addition, the N terminal part of Alix also shows strong homologies with the GTP-Rho binding protein Rhoophilin¹³ within its N terminal region (34% identity within aa 121–385 of Rhoophilin).

Tissue distribution of Alix

We next investigated Alix and ALG-2 tissue distribution by Northern blot analysis of different adult mouse tissues. As shown in Figure 2 Alix and ALG-2 are widely expressed in all tissues analyzed. As previously described, a 1.3 Kb band was detected for ALG-2.¹ Using Alix SF cDNA as a probe, we could detect two different transcripts of approximately 3.5 Kb and 6.5 Kb. A similar pattern was observed when we used a probe covering the central part of Alix cDNA (not shown). Since this region is missing in Alix SF cDNA, this suggests that none of the two transcripts correspond to the putative spliced variant of Alix cDNA.

Alix/ALG-2 and ALG-2/ALG-2 interactions are Ca²⁺-dependent and -independent, respectively

In order to confirm the Alix/ALG-2 and ALG-2/ALG-2 interactions detected in the yeast two hybrid system, we next performed co-immunoprecipitation experiments of over-expressed proteins in human embryonic kidney 293 cells (HEK 293 cells). Cells were transiently co-transfected with expression vectors coding for Myc-tagged Alix, Flag- or Myc-tagged ALG-2 or Flag-tagged sorcin, as indicated. Sorcin, which is the closest homologue of ALG-2 within the calmodulin family (39% identity) was used as a control. Twenty-four hours later, cells were lysed and immunoprecipitated using an anti-Myc monoclonal antibody. Co-precipitating proteins were revealed by immunoblotting using an anti-Flag monoclonal antibody. The effect of calcium on protein interactions was tested by performing lysis and immunoprecipitations in presence or absence of Ca²⁺.

Figure 3 shows that ALG-2 co-precipitated strongly with Alix in a buffer containing 5 mM CaCl₂. This interaction was strictly Ca²⁺ dependent since it did not occur when CaCl₂ was replaced by MgCl₂. Sorcin did not co-precipitate with Alix in presence or absence of Ca²⁺ demonstrating the specificity of the Alix/ALG-2 interaction.

A truncated Alix possessing 401 amino acids of the C-terminal region and corresponding to the cDNA isolated

using the yeast two hybrid system (Alix CT) could also precipitate ALG-2. In contrast, Alix lacking the C-terminal half (Alix NT) or most of the central part of the protein (Alix

SF) did not interact with ALG-2 (Figure 4). These results indicate that the region between aa 468 and 806 of Alix contains the domain of interaction with ALG-2.

a

b

Figure 1 Primary amino acid sequence of mouse Alix and comparison with *C. elegans* YNK-1. (a) Deduced amino acid sequence from the mouse Alix full-length cDNA. Amino acids identical in both mouse Alix and the *C. elegans* YNK-1 are boxed. The black box represents a tyrosine phosphorylation consensus site. The two horizontal arrows indicate the amino acids juxtaposed in the Alix short form sequenced from the mouse EST clone (accession number W97887). The SH3 binding motifs are boxed in grey. The first residue of the clone isolated from the yeast two hybrid screen is indicated by a filled vertical arrow. (b) Sequence relatedness of Alix and its homologues; lengths of the branches represent the average identities of the different members. The sequence alignment and dendrogram were computed with Laser Gene-Megalign software (DNASTar, Madison, WI, USA)

Figure 2 Alix mRNA distribution. Poly(A)⁺ RNA (2 µg) from different adult mouse tissues (Clontech) was probed with ³²P-labelled Alix and ALG-2 under high stringency conditions. Human β-actin was used as an internal standard control

Because Alix sequence contains no obvious Ca²⁺ binding motif, our results suggest that the interaction between Alix and ALG-2 occurs following the binding of Ca²⁺ to ALG-2. As with other EF hand protein, ALG-2 may act as a sensor of intracellular Ca²⁺, undergoing a conformational change upon Ca²⁺ binding to allow its interaction with Alix.

In contrast to the Ca²⁺ dependent interaction between Alix and ALG-2, ALG-2 precipitated with itself in presence or absence of Ca²⁺ (Figure 3). Within the calmodulin family, ALG-2 is a member of a subfamily including calpain, grancalcin, sorcin and an open reading frame in *S. cerevisiae*. All of these proteins contain 5 EF-hands (EF-1–5) and have been shown to bind Ca²⁺.⁵ Grancalcin and sorcin exist as homodimers. The Ca²⁺ binding domain of the small subunit of calpain has recently been shown to form homodimers in absence of the catalytic subunit. In this latter case, X-ray crystallography has revealed that one EF-5 pairs up with another EF-5 in a Ca²⁺ independent fashion.^{14,15} Our results indicate that the last 92 amino acids of ALG-2 which contain EF-5, EF-4 and part of EF-3 are sufficient to interact with ALG-2. This suggests that ALG-2 may also form dimers and raises the

Figure 3 Differential effects of Ca²⁺ on ALG-2/ALG-2 and Alix/ALG-2 interactions. HEK 293 cells were transiently co-transfected with expression vectors encoding Myc tagged or Flag tagged-Alix, -ALG-2 and -sorcin, as indicated. Cells were lysed in buffer containing either 5 mM CaCl₂ or 5 mM MgCl₂ together with 1 mM EGTA. Immunoprecipitations of Alix and ALG-2 were performed using an anti-Myc monoclonal antibody. Co-immunoprecipitated proteins were revealed using an anti-Flag monoclonal antibody (upper panel). Immunoprecipitated Alix and ALG-2 as detected using anti-Myc antibody are shown in the middle panels. The lower panel shows ALG-2 and sorcin proteins in the transfected cell lysates, as revealed by the anti-Flag antibody

question of the role of EF-5 in this Ca²⁺ independent homophilic interaction.

The function of Alix is today unknown. It is noteworthy that PalA is part of a signal transduction pathway mediating pH regulation of gene expression and thereby participating in ambient pH regulation in *A. nidulans*.¹¹ Nickas and Yaffe have shown that BRO1 interacts with components of the Pkc1p-mitogen-activated protein kinase pathway in yeast.¹² One may therefore hypothesise that members of the Alix family are involved in signal transduction regulated by Ca²⁺ and Ca²⁺ binding proteins such as ALG-2. The presence of proline rich sequences within the C-terminal region of these proteins may indicate that they bind to cytoskeletal and signal transduction proteins containing SH3 and WW domains. Using Alix as bait in a yeast two hybrid system may help to identify additional interacting proteins and thereby give an insight into the protein's function in the cell death program. These studies are currently underway in our laboratory.

Materials and Methods

Biological reagents and cell culture

The anti-Flag BioM2 monoclonal antibody was purchased from Eastman Kodak Company (New Haven, CT, USA). Anti-Biotin-POD monoclonal antibody was provided by Boehringer Mannheim Biochemica (Germany). Anti-Myc monoclonal as well as anti-Myc

polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human embryonic kidney 293 cells and Hela cells were maintained in DMEM/F12 medium (Gibco BRL) containing 10% Foetal Calf Serum, using standard cell culture conditions.

Yeast two hybrid cloning

ALG-2 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 DH5- α cells (Clontech). cDNAs were sequenced with a Perkin Elmer/ABI 373XL sequencer.

cDNA cloning

ALG-2 cDNA was amplified by RT-PCR using mouse brain RNA as template. The primers were designed according to the sequence published,¹ (primer 5'-GCCATGGGGATCCGTGTGCATCAGC-CCATGGCTG, primer 3'-AGGCCTGGTTATACAATGCTG). The PCR product was subcloned into the yeast expression vector pGBT9 and into the mammalian expression vector pCI (Promega) containing either Myc or Flag epitope tag. Sorcin cDNA was amplified by RT-PCR using human brain cortex RNA. The primers were designed using the sequence published¹⁶ primer 5'-AGGATCCATAGCATGGCGTACCCG-GGGCAT, primer 3'-AGGAGAGCTCCAGTTGGAATGTTGAT-TACA). The PCR product was subcloned into pCI with a Flag tag.

A full-length cDNA encoding mouse Alix was cloned into the Bluescript KS⁺ vector in three steps; (1), a 1700 bp *EcoRI* fragment subcloned in the pGAD10 vector (Clontech) was obtained by the two hybrid screen; (2), a 1707 bp insert, containing approximately 500 bp of overlapping sequences at the 5' end of the two hybrid product, was obtained by screening a λ gt10 mouse cDNA library (Clontech) and subcloned into the *EcoRI* site of Bluescript KS⁺ vector; (3), a 410 bp fragment, containing 209 bp of overlapping sequences with the former insert, was amplified by RT-PCR using mouse brain RNA as template and subcloned into the *SmaI* site of Bluescript KS⁺. The primers were designed using an EST sequence (accession number: W97887) (primer 5'-TCACCATGGCGTCGTTTCATC, primer 3'-TCTGCTGCAATCTGGCTAGC). The three fragments were ligated together into Bluescript KS⁺ using appropriate restriction enzymes.

Alix-NT was cloned by excising a *SaI* fragment of Alix full-length and corresponds to aa 1–434. Alix-CT (aa 468–869) is the product of the two hybrid screen. Alix-SF (aa 1–158 and aa 806–869) corresponds to the EST sequence described above. The different Alix cDNAs were all subcloned into pCI-Myc vector.

Northern blot hybridisation

Northern blot analysis of mouse tissues (Clontech) was performed according to the instructions of the manufacturer. The ALG-2 probe was the full-length cDNA. For Alix, we used either the Alix-SF cDNA or a 500 bp fragment corresponding to nucleotides 1403 to 1903.

Transfections, coimmunoprecipitations and Western blot analysis

HEK 293 cells at 50% confluence were co-transfected with a total of 10 μ g of DNA in 10 mm petri dishes by calcium phosphate

Figure 4 The COOH half part of Alix is necessary for its binding with ALG-2. (a) Schematic representation of Alix deletion mutants used in (b). The black boxes at the N terminus of the proteins represent the Myc tag. Numbers correspond to amino acid positions. (+) and (–) indicate the ability of the protein to interact with ALG-2. (b) Co-immunoprecipitations of Alix deletion mutants with ALG-2. HEK 293 cells were transiently co-transfected with Myc tagged-Alix WT, -Alix NT, -Alix CT, -Alix SF and Flag tagged-ALG-2. The green fluorescent protein (GFP) was used as a control. Cells were lysed in presence of 5mM CaCl_2 . Alix proteins were immunoprecipitated with the anti-Myc monoclonal antibody and immunodetected with an anti-Myc polyclonal antibody (middle panel). Co-immunoprecipitation of ALG-2 (upper panel) and cell lysates (lower panel) were revealed by immunoblotting with the anti-Flag monoclonal antibody

precipitation. After 24 h, cells were lysed in 500 μ l of either lysis buffer A (142.5 mM KCl, 5 mM CaCl_2 , 10 mM HEPES, 0.2% NP40, pH 7.4) or lysis buffer B (142.5 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 10 mM HEPES, 0.2% NP40, pH 7.4). Protease inhibitors were added according to the manufacturer protocol (complete, Mini EDTA-free, Boehringer Mannheim, Germany). Cellular debris were removed by centrifugation at $10,000 \times g$ for 10 min, and 300 μ l of the supernatants were pre-incubated 4 h at 4°C with 30 μ l of protein G-agarose (Boehringer Mannheim, Germany). After discarding the beads, the lysates were incubated for 16 h at 4°C with 1 μ g of anti-Myc monoclonal antibody. Thirty μ l of protein G agarose was then added to the antibody-containing-supernatants and incubated for a further hour at 4°C . The beads were then washed extensively with buffer A or B. Bound proteins were run on a 4–20% SDS-polyacrylamide gel (Novex, CA, USA) and transferred on nitro-cellulose membranes (Novex). Immunoblot analysis was performed using anti-Myc monoclonal or polyclonal antibody, or with anti-Flag Bio-M2 monoclonal antibody.

Note added in proof

Since the submission of this article, a novel putative protein tyrosine phosphatase (PTP-TD14; Genbank acc: AF077000) has been cloned which shows in its NH2 terminal part high homologies with Alix (25% identity on 650 aa), YNK-1, PalA and Bro1 (Cao *et al.* (1998) *J. Biol. Chem.* 273: 21077–2183). Therefore this putative phosphatase which suppresses Ha-ras mediated transformation may be the first member of the Alix family with an enzymatic activity.

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