Two Forms of the Apoptosis-linked Protein ALG-2 with Different Ca\(^{2+}\) Affinities and Target Recognition*  

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The apoptosis-linked gene ALG-2 encodes a Ca\(^{2+}\)-binding protein of the pentα EF-hand family. To investigate the Ca\(^{2+}\) binding properties of the recombinant ALG-2 protein, we have cloned ALG-2 cDNA from mouse liver mRNA. Sequence analysis showed that two types of clones were present. One (named ALG-2,5) corresponds to the published ALG-2 sequence (Vito, P., Lacana, E., and D’Adamio, L. (1996) Science 271, 521–525); the second (named ALG-2,1) is 6 nucleotides shorter, and the corresponding protein lacks the amino acid residues Gly\(^{121}\) and Phe\(^{122}\). Both transcripts are present in mouse tissues in the same 2:1 molar ratio. The ALG-2,5 and ALG-2,1 recombinant proteins are fully soluble in the metal-free form but can be precipitated from bacterial lysates by Ca\(^{2+}\). In the presence of Tween the Ca\(^{2+}\)-binding profiles display two high affinity sites with [Ca\(^{2+}\)]\(_{0.5}\) values of 1.2 and 5.1 \(\mu\)M for ALG-2,5 and ALG-2,1, respectively, plus one low affinity site. Using the yeast two-hybrid system we demonstrate that both proteins have a strong tendency to form homo- and heterodimers. In contrast to ALG-2,5, the ALG-2,1 isoform does not interact with the target protein AIP-1, earlier described to play a role in apoptosis (Vito, P., Pellegrini, L., Guiet, C., and D’Adamio, L. (1999) J. Biol. Chem. 274, 1533–1540). We propose that the minor sequence difference between ALG-2,5 and ALG-2,1 affects the Ca\(^{2+}\) binding properties and function of the proteins.

In many experimental systems it has been shown that an increased intracellular Ca\(^{2+}\) concentration, generated for instance by ionophores, induces apoptotic cell death (1). Transiently elevated Ca\(^{2+}\) concentrations are required for several pathways leading to apoptosis such as glucocorticoid-mediated (2) and T-cell receptor-mediated (3) T-cell death. Removal of extracellular Ca\(^{2+}\) or buffering of intracellular Ca\(^{2+}\) can prevent DNA fragmentation and apoptotic body formation (1). Ca\(^{2+}\)-dependent nuclease seem to be involved in apoptosis (4, 5). It has been demonstrated that calmodulin (6) and its target proteins such as calcineurin (7, 8), Ca\(^{2+}\)-dependent kinases (9–11), as well as the Ca\(^{2+}\)-dependent protease calpain (12) play important roles in programmed cell death. However, the molecular pathways triggering Ca\(^{2+}\)-dependent apoptosis have been poorly investigated.

One promising mediator of Ca\(^{2+}\) regulated apoptosis, the ALG-2 protein, was discovered in a “death trap” assay using T-cell receptor-mediated apoptosis as a model system (13). Transfection of a variety of cells including T-cells, fibroblasts, and epithelial cells with an antisense ALG-2 construct inhibits apoptosis induced by several stimuli such as staurosporin and dexamethasone. Because in the ALG-2 depleted clones protected from apoptosis caspases were normally activated following T-cell receptor engagement, it was concluded that the ALG-2 protein acts either downstream of caspases or in a caspase-independent way (14). ALG-2 expression is not enhanced in apoptotic cells, indicating that its activity is regulated by protein modification or by ligand interaction. The ALG-2 protein was highly conserved during evolution with 70% of sequence identity between human and nematodes (15).

From sequence comparisons it appeared that the ALG-2 protein contains five EF-hands, with two of them being functional in \(^{45}\)Ca\(^{2+}\) overlay experiments (13), and furthermore it shares homology with members of the PEF (penta EF-hand) family, which includes sorcin (16), grancalcin (17), calpain light chain (18), yeast hypothetical protein of 38.4 kDa (19), and peflin (20). These proteins contain a glycine-rich N-terminal region proposed to play an important role in Ca\(^{2+}\)-dependent membrane binding (19). At high protein concentrations recombinant ALG-2 can be precipitated by Ca\(^{2+}\) (21). The nonionic detergent Triton X-100 (1%) prevents this precipitation, suggesting an involvement of hydrophobic interactions in the Ca\(^{2+}\) induced precipitation. Indeed ALG-2 exhibits a Ca\(^{2+}\)-dependent exposure of a hydrophobic surface as monitored with a fluorescent hydrophobicity probe (21). Thus, it is likely that ALG-2 activation is initiated by Ca\(^{2+}\) binding and may lead to hydrophobic interactions with target(s). Recently, two reports on the interaction of ALG-2 with a target protein either named Alix (22) or AIP-1 (23) were published. Alix/AIP-1 interaction with ALG-2 was shown to be Ca\(^{2+}\)-dependent in the submicromolar range. Because truncated AIP-1, which is still able to bind ALG-2, can block apoptosis, it was postulated that AIP-1 cooperates with the ALG-2 protein to mediate the Ca\(^{2+}\)-dependent signaling leading to cell death. Here we report that two transcripts of ALG-2 are present in mouse tissues. We have expressed the two ALG-2 isoforms in Escherichia coli and compared several biophysical properties of the purified proteins. Despite the difference of only 2 amino acids, the ALG-2 isoforms differ significantly in their Ca\(^{2+}\) binding properties and in their ability to interact with AIP1.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of the Recombinant ALG-2 Protein—Total RNA was isolated from freshly prepared adult mouse...
liver according to the method of Chomczynski and Sacchi (24). cDNA was synthesized using oligo(dT) as a primer with standard procedures. This cDNA served as a template for PCR using primers containing either the ALG-2 ATG start codon or its translation TAA stop codon with restriction sites XbaI (5'-AGCTTCACTGGGTCGAC-3') and EcoRI (5'-GGCTGCTGCATCTGTTTGTTGCT-3'). Primer 2 (TAA), 5'-GCCGCGTTGCCTGTTCTCAAGAAGCCTGTT-3'. Primer 1 contains an NdeI site (underlined), and primer 2 contains a BamHI site (underlined). The PCR product was digested with NdeI and BamHI followed by ligation with pGEMEXII (Promega). The ALG-2 insert of several clones was sequenced by the dideoxy chain termination method (25). Both ALG-2 probes were expressed in the E. coli, strain BL-21(DE3). Expression of the ALG-2 probes was induced at A600 0.7 by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The bacteria were harvested after 2.5–3 h by centrifugation at 8400 × g for 15 min, resuspended in 0.1 vol (of bacteria culture) of a buffer containing 2.4 M sucrose, 40 mM Tris-HCl, pH 8, and 10 mM EDTA and incubated at 4 °C for 30 min. Lysis was carried out at 4 °C overnight by the addition of 0.04 volume of a solution containing 50 mM HEPES, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.1 mg/ml lysozyme (Sigma), 1 mM dithiothreitol. The lysate was clarified by centrifugation at 34,500 × g for 30 min. The ALG-2 protein was then precipitated from the supernatant by addition of 5 mM CaCl2 on ice for centrifugation by centrifugation at 17,200 × g for 15 min. The precipitate was washed with 20 mM Tris-HCl, pH 8, 1 mM CaCl2 and dissolved in 0.01 vol of a buffer containing 5 mM Tris-HCl, pH 8, 20 mM EDTA, 20 mM Tris-HCl, pH 8, at 4 °C.

The supernatant after a further centrifugation step (17,500 × g for 15 min) was subjected to anion exchange chromatography using a PE 4.6/100 (Amersham Pharmacia Biotech) column and an Aekta (Amersham Pharmacia Biotech) chromatographic system. Following injection of the sample (up to 50 ml) the column was washed with one sample volume of buffer A (20 mM Tris-HCl, pH 8, 1 mM EDTA) followed by a gradient from 0 to 35% buffer B (buffer A + 500 mM NaCl) in totally 22 ml. The column was finally washed with 100% buffer B. The protein concentration was estimated by the Lowry colorimetric test (26). The purity of the proteins was analyzed by 12% polyacrylamide gel electrophoresis.

**Molecular Mass and Secondary Structure Determination**—Metal-free protein samples desalted by Sephadex G-25 gel filtration in 5 mM ammonium bicarbonate and subsequently lyophilized were subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with myoglobin and aldolase as internal standards.

**RESULTS AND DISCUSSION**

Cloning and Sequencing of ALG-2—Because ALG-2 transcripts are present in relatively high abundance in mouse liver (13), ALG-2 cDNA was cloned by PCR amplification using liver mRNA as a template. The PCR product containing the complete coding sequence was cloned into the bacterial expression vector pGEMEXII. Sequencing of several clones revealed that two forms of ALG-2 transcripts exist (Fig. 1). One, named ALG-2,5, was identical to the published sequence (13), whereas the second (ALG-2,1) lacked 6 base pairs corresponding to the amino acid residues Gly82 and Phe124 in the linker region between the 3rd and 4th EF-hand of the ALG-2,5 protein. 10 clones were further analyzed by PCR with primers adjacent to the six differing base pairs. Five clones gave rise to a PCR product of 55 bp and 5 clones yielded in 61 bp indicative of the ALG-2,1 and ALG-2,5 forms (results not shown). Further support for the existence of two ALG-2 isoforms was provided by the presence of both sequences in human and mouse EST DNA data banks.

**Two ALG-2 Transcripts Are Present in Mouse Tissues**—In preliminary experiments using Northern blot analysis, we have shown that brain, kidney, and ovaries express high amounts of ALG-2 RNA (not shown). To investigate whether indeed two transcripts are present in these tissues the RNase protection assay was carried out. An ALG-2 specific probe was designed to

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1 The Abbreviations used are: PCR, polymerase chain reaction; nt, nucleotides(s); bp, base pairs.

**Isoforms of the Ca**

**2**

**-binding Protein ALG-2**

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protect a 263-nucleotide fragment of the ALG-2,5 transcript and two fragments of 194 and 63 nucleotides, respectively, corresponding to the ALG-2,1 transcript. We found that both RNA species are transcribed (Fig. 2) in a molar ratio of 2:1 (ALG-2,5 to ALG-2,1) as monitored by PhosphoImager scanning.

Purification of the ALG-2,1 and ALG-2,5 Recombinant Proteins—Initial experiments revealed that the recombinant ALG-2 proteins can be selectively precipitated by millimolar Ca\(^{2+}\) concentrations from bacterial lysates. Ca\(^{2+}\)-dependent precipitation was therefore used for the purification of the recombinant proteins. The proteins were precipitated twice with 5 mM CaCl\(_2\) followed by anion exchange chromatography in a salt gradient (Fig. 3). This led to a high purity of the ALG-2 proteins, with a final yield of 5–10 mg of purified protein/liter of bacterial culture. Amino acid analysis was carried out to verify the protein preparations.

The experimental values of the molecular masses determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry were 21,533 ± 6 and 21,739 ± 9 for ALG-2,1 and ALG-2,5, respectively. These values correspond precisely to the theoretical molecular masses of the proteins, whose N-formyl Met is post-translationally cleaved off from the N terminus (theoretical values are 21,532 and 21,736 for ALG-2,1 and ALG-2,5, respectively). The two isoforms have the same mobility in SDS-containing gels and display a molecular mass of 22 kDa. Their UV spectra are slightly different but show the abundance of Trp and Tyr residues (data not shown). A molecular extinction coefficient at 278 nm of 39,200 M\(^{-1}\) cm\(^{-1}\) was used to determine the protein concentration for both isoforms.

Secondary Structure—In the presence of 0.5% Tween neither of the isoforms precipitates upon addition of Ca\(^{2+}\) at protein concentrations up to 50 \(\mu\)M. Circular dichroism on the metal-free and Ca\(^{2+}\)-loaded forms of ALG-2,1 and ALG-2,5 in the presence of this detergent showed that the proteins display similar far UV spectra (data not shown). The results of the secondary structure predictions according to Ref. 27 are summarized in Table I. Ca\(^{2+}\) binding or dissociation only slightly modify the secondary structure of both ALG-2 isoforms, a feature also observed in several other Ca\(^{2+}\)-binding proteins, such as calretinin (30) or S100 proteins (31).
Isoforms of the Ca\textsuperscript{2+}-binding Protein ALG-2

![Graph: Flow dialysis to determine Ca\textsuperscript{2+} affinities.](image)

Fig. 4. Flow dialysis to determine Ca\textsuperscript{2+} affinities. Ca\textsuperscript{2+} binding was measured (in triplicate) by flow dialysis at 25 °C in 50 mM Tris-HCl, pH 7.5, 150 mM KCl. Rectangles, ALG-2,1; circles, ALG-2,5. Open symbols, no additional ions; closed symbols, 2 mM Mg\textsuperscript{2+}. Experiments were also carried out in the presence of 0.5% Tween. Hexagons, ALG-2,1; stars, ALG-2,5. The lines connecting the symbols were generated using Adair equation with the following intrinsic constants: (K\textsuperscript{D}: 1.7 × 10\textsuperscript{4}, 3.8 × 10\textsuperscript{4}, and 1.1 × 10\textsuperscript{5} M\textsuperscript{-1} for ALG-2,1; 1.0 × 10\textsuperscript{4}, 1.0 × 10\textsuperscript{5}, and 3.0 × 10\textsuperscript{4} M\textsuperscript{-1} for ALG-2,5; 1.8 × 10\textsuperscript{3}, 1.8 × 10\textsuperscript{3}, and 3.0 × 10\textsuperscript{3} M\textsuperscript{-1} for ALG-2,1 in the presence of 0.5% Tween and 4.5 × 10\textsuperscript{3}, 4.5 × 10\textsuperscript{3}, and 3.0 × 10\textsuperscript{3} M\textsuperscript{-1} for ALG-2,5 in the presence of 0.5% Tween.

![Table: Growth on medium lacking histidine](image)

<table>
<thead>
<tr>
<th>Growth on medium lacking histidine</th>
<th>ß-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 + SV40</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>BD ALG-2,1 + AD ALG-2,1</td>
<td>121.8 ± 8.9</td>
</tr>
<tr>
<td>BD ALG-2,5 + AD ALG-2,5</td>
<td>106.2 ± 16.6</td>
</tr>
<tr>
<td>BD ALG-2,5 + AD GAL4</td>
<td>116.7 ± 12.6</td>
</tr>
<tr>
<td>BD ALG-2,1 + AD ThH2B</td>
<td>0.135 ± 0.04</td>
</tr>
<tr>
<td>BD ALG-2,5 + AD ThH2B</td>
<td>8.16 ± 1.6</td>
</tr>
<tr>
<td>BD ALG-2,1 + AD GAL4</td>
<td>0.159 ± 0.02</td>
</tr>
<tr>
<td>BD ALG-2,5 + AD GAL4</td>
<td>0.220 ± 0.06</td>
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</tbody>
</table>

Fig. 5. Two-hybrid analysis of ALG-2 dimerization and target recognition. Yeast clones co-transfected with the GAL4 DNA-binding domain (BD) and GAL4 transcriptional activation domain (AD) hybrid constructs were first grown on SD medium without Trp and Leu (selection for the plasmids) and then streaked onto a SD plate lacking histidine. The HIS3 phenotype was analyzed after 3–5 days. Each patch represents an independent transformant. The ß-galactosidase activity was determined for at least three independent clones from each transformation. Values (mean ± S.D.) are given in arbitrary units. The p53 and SV40 plasmids were used as a positive control for the two-hybrid assay. Yeast transfected with either the BD ALG-2,1 or BD ALG-2,5 plasmids together with the AD GAL4 empty vector served as negative controls.

two marked differences concerning the high affinity sites: (i) the Ca\textsuperscript{2+} sensitivity of ALG-2,5 is higher than that of ALG-2,1 with a [Ca\textsuperscript{2+}]\textsubscript{0.5} value of 1.2 versus 3.1 µM, respectively, and (ii) the Hill coefficient, n\textsubscript{H}, of the ALG-2,5 curve is 1.1, that of ALG-2,1 is 1.9.

In the absence of detergent the proteins consistently precipitate during the titration, thus demonstrating an increase in the complexity of the binding phenomena. The isotherms are quite different (Fig. 4, rectangles and circles); ALG-2,1 displays a single set of three sites with a [Ca\textsuperscript{2+}]\textsubscript{0.5} of 28 µM and n\textsubscript{H} = 1.6, and ALG-2,5 possesses two sites with a [Ca\textsuperscript{2+}]\textsubscript{0.5} of 5.5 µM and n\textsubscript{H} of 2.0, plus one site with a K\textsubscript{D} of 330 µM. Thus, the precipitation perturbs the Ca\textsuperscript{2+} binding properties of ALG-2,1 much more than those of ALG-2,5. The binding isotherms are not affected by the presence of 2 mM Mg\textsuperscript{2+}, indicating that the functional EF-hands in ALG-2 are of the Ca\textsuperscript{2+}-specific type as found in calmodulin (32).

Note that in the presence of Tween the high affinity sites have a 5–10-fold higher affinity for Ca\textsuperscript{2+} than in the absence of the detergent. The increased affinity in the presence of detergent likely reflects the fact that the energetically unfavorable exposure of hydrophobic patches to the polar solvent is mostly abolished if the detergent occupies these hydrophobic patches.

In conclusion, both ALG-2 isoforms show Ca\textsuperscript{2+} affinities in the low micromolar range. This is comparable with values found for Ca\textsuperscript{2+} modulated proteins, such as calmodulin (10), which serve as mediators in intracellular signal transduction. Therefore, our results indicate that ALG-2 has a potential to act in Ca\textsuperscript{2+} triggered pathways leading to apoptosis. Interestingly, ALG-2,1 has a lower Ca\textsuperscript{2+} affinity than ALG-2,5, indicating that the two proteins might be recruited during Ca\textsuperscript{2+} transients in a different fashion.

From the primary structure it is obvious that EF-hands 1 and 3 are the canonical ones and that all other sites contain aberrations. Vito et al. (13) studied Ca\textsuperscript{2+} binding to deletion mutant proteins that contained either site 1 or 3 and observed that efficient Ca\textsuperscript{2+} binding occurs only if both EF-hands are present. Lo et al. (33) inactivated site 1, site 3, or both by point mutations and demonstrated that Ca\textsuperscript{2+} binding induced conformational changes in each of the single mutant proteins but not in the proteins with two mutated sites. Combining these results with our direct binding data, we suggest that EF-hands 1 and 3 are the high affinity sites in the ALG-2 isoforms. The low affinity site is more difficult to localize because both sites 2 and 4 contain a positively charged residue in the Z position, a Ca\textsuperscript{2+}-coordinating position, which normally provides an oxygen atom from a side chain carbonyl group. EF-hand domain 5 contains an insertion of two amino acid residues and glutamine in the –Z position, which is not generally found in functional EF-hands. Future mutational analysis will allow a precise
localization of the sites and an evaluation of their importance for the function of the ALG-2 proteins.

In the absence of available structural information the difference in Ca\(^{2+}\) binding properties of the ALG-2 isoforms is difficult to explain. The amino acid residues Gly\(^{121}\) and Phe\(^{122}\) are located in the linker region beyond the 3\(^{rd}\) EF-hand, thus the lack of these residues in the ALG-2,1 protein cannot directly affect the Ca\(^{2+}\)-binding site. More likely this “deletion” causes some conformational changes in the ALG-2,1 protein, which might be critical for intramolecular interactions and EF-hand integrity resulting in lower Ca\(^{2+}\) affinity as compared with ALG-2,5. However, the final conclusion can be made only based on the three-dimensional structure of the ALG-2 isoforms.

Dimerization of ALG-2 in Vivo—It is well documented that proteins of the PEF family can form homodimers (34, 35) or heterodimers (36). ALG-2 contains an abortive fifth EF-hand motif that functions as a dimerization site in the related protein, calpain light chain (18). Lo et al. (33) have recently shown by gel filtration chromatography and chemical cross-linking that ALG-2,5 can occur in a monomeric or dimeric form. The dimerization of ALG-2,5 was detected by the two-hybrid screening of a mouse cDNA library (22). Here, we have applied the yeast two-hybrid system (37) to investigate homo- and heterodimerization of the ALG-2 isoforms. Both, ALG-2,5 and ALG-2,1 cDNA were cloned in-frame with either the GAL4 upstream activation sequence. The interaction phenotype was scored by the reporter genes under the control of the GAL4 upstream activation domain, calpain light chain (18). Lo et al. (33) have recently shown that ALG-2,5 can occur in a monomeric or dimeric form. The dimerization of ALG-2,5 was detected by the two-hybrid screening of a mouse cDNA library (22). Here, we have applied the yeast two-hybrid system (37) to investigate homo- and heterodimerization of the ALG-2 isoforms. Both, ALG-2,5 and ALG-2,1 cDNA were cloned in-frame with either the GAL4 DNA-binding domain or the transcriptional activation domain. The hybrid proteins were expressed in different combinations in the YRG-2 yeast strain harboring integrated lacZ and HIS3 reporter genes under the control of the GAL4 upstream activating sequence. The interaction phenotype was scored by the ability of the transformed yeast to grow on a synthetic medium without histidine and by β-galactosidase activity (Fig. 5). We found that both ALG-2 isoforms form homo- and heterodimers with similar and rather high efficiency as compared with the interaction between p53 and SV40 large T-antigen, which served as a positive control.

Differential Target Recognition by the Two ALG-2 Isoforms—Despite the fact that ALG-2 was cloned by functional screening (13), the molecular mechanism and mode of regulation of its action still remain unknown. So far, one target protein named AIP1 (23) or Alix (22) was identified in a two-hybrid screen. AIP1 (23) or Alix (22) was identified in a two-hybrid screen. AIP1 cDNA. We also thank Christian Hansen for construction of the pdTh28 plasmid and Dr. J. Mollerup for critical reading of the manuscript.

Acknowledgments—Dr. P. Vito is acknowledged for providing us with AIP1 cDNA. We also thank Christian Hansen for construction of the pdTh28 plasmid and Dr. J. Mollerup for critical reading of the manuscript.

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