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ALG-2, A MULTIFUNCTIONAL CALCIUM BINDING PROTEIN?

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1. ABSTRACT

ALG-2 was originally discovered as a pro-apoptotic protein in a genetic screen. Due to its ability to bind calcium with high affinity it was postulated to provide a link between the known effect of calcium in programmed cell death and the molecular death execution machinery. This review article discusses the current knowledge on the structure and potential function of this protein. Several putative binding partners of ALG-2 have been identified hinting to functions of ALG-2 in apoptosis and possibly also in proliferation, endocytosis and transcriptional regulation during development. Gene deletion of the well conserved ALG-2 locus in several genetic model organisms has so far not provided insights into functions and signaling pathways with ALG-2 involvement. Special focus is given to controversial data on expression and localization of ALG-2, which is mainly caused by the use of ALG-2 antibodies with different specificities.

2. INTRODUCTION

In the mid 1990´s several groups developed functional screening procedures in order to discover genes with relevance in apoptotic processes using a variety of cell types and apoptosis inducing agents (1-3). Vito *et al.* (4) found by such a functional selection strategy, called death trap assay by the authors, that the products of two genes, Apoptosis-Linked Genes 2 and 3, ALG-2 and ALG-3, interfere with apoptosis of 3DO mouse T-cell hybridomas induced by T-cell receptor (TCR) cross-linking. Whereas ALG-2, an EF-hand type calcium binding protein, seemed to have a pro-apoptotic function ALG-3, a truncated form of the familial Alzheimer´s disease gene presenilin 2, rescued T cells from apoptosis induced by cross-linking of the TCR or Fas receptor.

Molecular mechanisms of calcium-induced apoptosis are not well established (5) although it is clear that calcium signaling plays an important role in a variety of apoptotic pathways, one of them being TCR induced apoptosis (6, 7). Therefore, ALG-2, a novel member of the penta-EF-hand (PEF) family, a protein with pro-apoptotic function and calcium binding properties (4) seemed to be a good candidate molecule for the missing link between calcium signaling and execution of the apoptotic program. This prompted a series of further studies on ALG-2, mostly on a biochemical level (8-10). Several review articles exist on PEF family proteins describing these findings (11-13). The goal of this review article is to give an overview on the ALG-2 research field with special emphasis on controversial aspects and new findings indicating that ALG-2 might have other functions than the apoptotic one.

3. ALG-2 IS A HIGH AFFINITY CALCIUM BINDING PROTEIN OF THE PEF FAMILY

ALG-2 is a 22 kD calcium-binding protein, which belongs to the PEF family comprising also calpain

large and small subunits, sorcin, grancalcin, peflin and a yeast hypothetical protein of 38.4 kD, Ygr058wp (11). Similar to calmodulin, PEF proteins utilize 12 amino acid residue long EF-hand loops to coordinate calcium ions (reviewed in (14)). Based on sequence analysis it was expected that only two out of five EF-hands of the ALG-2 protein are functional. Indeed, EF1 and EF3 – the only canonical EF-hands in the protein – were shown to bind $^{45}Ca^{2+}$ in overlay experiments (4, 9).

Two isoforms of the protein, ALG-2 and ALG-2.1, resulting from alternative splicing, exist (10). The ALG-2.1 mRNA is 6 nt shorter and the corresponding protein lacks the amino acid residues Gly^{121} and Phe^{122} in the linker region between EF3 and EF4. Despite of the fact that these two residues are not directly involved in calcium coordination, the ALG-2 isoforms differ in their calcium-binding affinities. Flow dialysis experiments have revealed that ALG-2 and ALG-2.1 bind three calcium ions per monomer of the protein, however, with different affinities (10). In the presence of 0.5% Tween, which allows to keep the protein soluble, both isoforms display one similar low affinity site (Kd = 300 μM), and two high affinity sites with Kd (Ca^{2+}) values of 1.2 and 3.1 μM for ALG-2 and the ALG-2.1 isoform, respectively. The difference in calcium affinities of the ALG-2 isoforms lies in the range of concentrations where calcium transients in the cell occur. Therefore, the two isoforms may be differentially recruited. All three sites seem to be of the calcium specific type similar to those found in calmodulin, since the presence of magnesium ions does not alter the shape of the Ca^{2+} binding isotherms.

3.1. Ca^{2+}-dependent conformational changes

Recombinant ALG-2 can be precipitated from solution by adding calcium at millimolar concentration. This unique feature of the protein allowed the development of a procedure for rapid and convenient purification of the recombinant ALG-2 (8-10). The precipitation effect was found to be concentration dependent and occurred quantitatively at ALG-2 concentrations greater than approximately 5 μM, while at 1 μM at least half of ALG-2 remained in the supernatant. The addition of Triton X-100 diminished the protein aggregation at high ALG-2 concentrations, indicating involvement of hydrophobic interactions in the aggregation process (8). Therefore, it was concluded that calcium binding induces conformational changes in the ALG-2 protein resulting in the exposure of hydrophobic surfaces, which interact to form aggregates.

Conformational changes in response to calcium binding are a characteristic feature of the EF-hand calcium binding proteins, such as calmodulin, troponin C and S100 proteins, involved in intracellular signal transduction. Upon calcium transients in the cell these proteins bind calcium ions and expose hydrophobic surfaces which allows interaction and modulation of the activity of the target proteins. In contrast, EF-hand proteins with mixed calcium/magnesium binding properties, such as parvalbumin, do not significantly change their conformation in response to ion binding and function mainly as calcium buffers in the cell (15, 16). Assuming that ALG-2 is a protein involved in intracellular signal transduction, it is expected to undergo a calcium-induced conformational change. A series of biophysical studies has been performed by several groups to address this question. Using TNS as a hydrophobicity probe (8) it was shown that addition of calcium indeed induced the exposure of hydrophobic surfaces in the ALG-2 protein. Notably, this conformational change did not exactly correlate with the calcium-dependent precipitation (8), as the half maximal effect of TNS occurred at 6 μM calcium, whereas the half maximal effect on the precipitation behaviour occurred at 20 μM calcium.

We have applied intrinsic tryptophan fluorescence measurements to monitor ALG-2 conformational changes upon calcium binding and obtained results similar to those reported by Maki *et al.* (8). Binding of calcium to ALG-2 at 3-5 μM protein concentration induced a blue shift and a drastic increase in fluorescence intensity, indicating that the surroundings of the tryptophan residues became more hydrophobic. This conformational change was dependent on the calcium concentration and was fully reversible by the addition of EGTA (Tarabykina, unpublished). Similar to Maki *et al.*'s data (8), this effect was accompanied by precipitation of the protein. The calcium concentrations required to achieve half maximal effects on the fluorescence change were lower than those required for protein aggregation, indicating that a hydrophobic conformational change preceded and perhaps facilitated precipitation. Interestingly, the calcium concentrations required for both effects were 10 times lower in the case of the ALG-2.1 isoform (Tarabykina, unpublished). In contrast to these data, Lo *et al*. (9) have reported that at ALG-2 concentrations where calcium-induced protein aggregation does not occur to a significant degree (below 1 μM) addition of calcium quenched the tryptophan fluorescence. Quenching continued as a consequence of calcium addition to the ALG-2 sample up to and exceeding a concentration of 10 mM, which was interpreted by the authors as a phenomenon indicative of the presence of weak calcium binding sites in the protein. However, the fluorescence changes were not reversible by addition of EGTA. This result was intriguing. Therefore, we have attempted to reproduce the experiment at the conditions which were used in the Lo *et al*. article (9) and came to the conclusion that fluorescence quenching was rather due to the sample dilution than to the conformational change in the protein, since addition of water or EDTA instead of calcium produced the same effect (Tarabykina, unpublished results).

Taken together, these data indicate that ALG-2 is a calcium-modulated protein, which is capable of responding to changes in calcium concentrations by undergoing conformational changes, and therefore may transmit calcium signals within the cell. This is further supported by the finding that calcium is required for interaction of ALG-2 with its target protein AIP1/Alix (17, 18), which is reminiscent of the interaction of calmodulin with the majority of its binding partners (19).

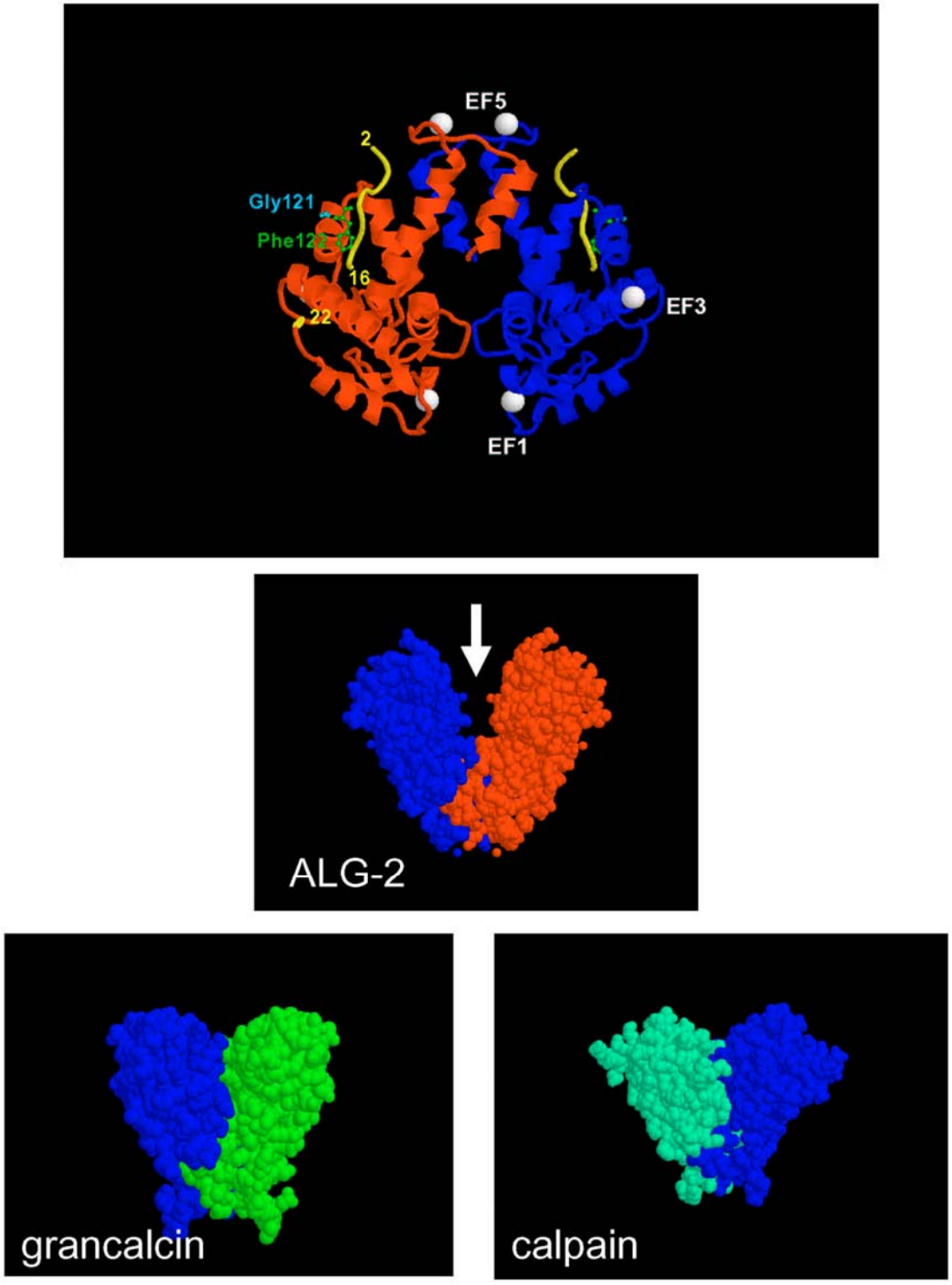

Figure 1. Structure of ALG-2 and comparison to other PEF-proteins in the calcium bound form. ALG-2 dimeric structure (20) (top panel): The two subunits are shown in red and blue colours, Gly 121 (cyan) and Phe 122 (green) are the residues not found in the short splice form of ALG-2 (10). Phe 122 is in close proximity to the N-terminal peptide (residues 2 – 16, yellow) which is bound to the ALG-2 after elastase cleavage C terminal of residue 21. The calcium ions bound to EF hands 1,3 and 5 are shown in white. Comparison of structures of ALG-2 (20), grancalcin (23) and calpain light chain (87) (middle and bottom panel). The subunits are shown in different colors. The arrow indicates the cleft in the ALG-2 structure between the subunits which is not present in the two other proteins.

4. THE THREE DIMENSIONAL STRUCTURE OF ALG-2 DIFFERS FROM THAT OF OTHER PEF PROTEINS

The three-dimensional crystal structure of the calcium-loaded ALG-2 protein has revealed a dimeric fold of ALG-2 (20) (Figure 1). Similar to sorcin and grancalcin, the ALG-2 monomer consists of a single domain of eight α-helices, which form five helix-loop-helix motifs known as EF-hand domains. These domains associate in a typical manner into pairs where EF1 pairs with EF2 and EF3 pairs with EF4. EF-hand 5 interacts with its counterpart in the other ALG-2 monomer to form a dimeric molecule.

In agreement with flow dialysis data (10) the crystal structure of ALG-2 revealed three calcium ions bound per monomer of ALG-2 (20) located in EF1 and EF3, which were previously predicted to be high-affinity sites based on sequence analysis (4, 8) and site directed mutagenesis (9). The third, low affinity, calcium-binding site was unexpectedly found at EF-hand 5, which is 2 amino acid residues longer than the canonical motif (21). It is questionable whether calcium binding to EF-hand 5 in ALG-2 is of physiological relevance.

One of the prominent features of the penta-EF-hand proteins is the presence of a proline/glycine rich region of variable length at the N-terminus. Such sequences have been proposed to play a role in calcium-dependent membrane binding (11) and target interactions (20). The attempts to crystallize an intact ALG-2 protein containing this domain failed. Therefore, the protein was subjected to limited proteolysis with elastase, which removed amino acid residues 1 to 20 as confirmed by N-terminal sequencing. Consequently, the crystallized ALG-2 protein comprised residues 21 to 191 (referred to des1-20 ALG-2 in (20)). After fitting the entire des1-20 ALG-2 a continuous electron density was found, the shape of which resembled a molecule without long branch points, i.e., presumably the ALG-2 N-terminal peptide (P_8GPGGGPGPA_{17}) cleaved from the protein with elastase. This putative peptide was found bound to a large hydrophobic cleft of des1-20 ALG-2 between the helix alpha7 and the loop connecting EF3 and EF4. Interestingly, Phe^{122}, the amino acid residue lacking in the short ALG-2.1 isoform, seems to form a direct contact with the peptide (Figure 1A). The interaction of the peptide with des1-20 ALG-2 involves hydrophobic as well as polar contacts and hydrogen bonding thus tightly binding it in a way as is utilized for binding of SH3 domains to their proline-rich targets (22).

The overall structure of the calcium loaded ALG-2 with a bound peptide differs significantly from that of sorcin, calpain and grancalcin both with and without calcium (Figure 1B) and suggests a mechanism involving domain movement (20): while the N-terminal and C-terminal halves of these molecules are similar, their relative disposition differs in ALG-2. It was speculated that this difference may be due to the presence of a peptide, whose binding requires movement of EF hands 1 and 2 relative to the C-terminus and the dimer interface as compared to the conformation found in grancalcin and sorcin. Comparison of the calcium bound and apo-grancalcin structures shows that calcium binding per se triggers only a very minor conformational change in the molecule (23). It was suggested that calcium ions are not likely to cause major conformational changes in the ALG-2 molecule either, but presumably initiate local conformational changes, which may be necessary for peptide binding. In turn, peptide binding may trigger a large conformational change in the ALG-2 molecule. A similar mechanism was previously described for the calcium-dependent binding of calmodulin to target peptides (24, 25).

5. ALG-2 FORMS STABLE HOMODIMERS

While the crystal structure clearly has shown that ALG-2 is able to form dimers, there are some contradictory data concerning the dimerization ability of ALG-2 in solution. Maki *et al*. (8) reported that in the presence of 1 mM EDTA ALG-2 existed as a monomer as seen by gel-filtration chromatography. Lo and co-authors (9) showed by analytical gel-filtration chromatography that both calcium-bound and apo-ALG-2 have a molecular weight of 22 kD indicating that ALG-2 is a monomer under the conditions used. However, upon applying a cross-linking technique, in the absence of calcium, they demonstrated the presence of dimers along with the ALG-2 monomers, which made the authors suggest that ALG-2 is a weak dimer in solution. When cross-linking was performed in the presence of calcium, the protein formed higher order aggregates incapable of migrating into the SDS-gel. Furthermore, the dynamic light scattering technique was applied to characterize the conformational state of ALG-2 in solution (unpublished data by Dr. I. Bronstein, with the kind permission of the author). These experiments revealed the exclusive presence of dimers with no detectable traces of monomers in solution both, in the presence and in the absence of calcium. ALG-2 dimerization was also analysed *in vivo* using the yeast two-hybrid system. Initially, the ALG-2 dimerization was found in the screen for ALG-2 interacting proteins by P. Vito *et al*. (17) and Missotten *et al*. (18). We have further characterized the dimerization of ALG-2 *in vivo* and have shown that both ALG-2 isoforms, ALG-2 and ALG-2.1, indeed can form stable homo- and heterodimers (10).

6. HETERODIMERIZATION OF ALG-2 AND STRUCTURAL REQUIREMENTS FOR TARGET INTERACTION

Heterodimerization seems to be a characteristic feature of the penta-EF-hand proteins, as all PEF-family members have the potential to heterodimerize with one another: ALG-2 was shown to heterodimerize with peflin, a protein with so far unknown function (26). Calpain large subunit binds the small subunit (reviewed in (27)), and grancalcin interacts with sorcin (28). Whereas the formation of the first two mentioned heterodimers only occurs in the absence of calcium, the heterodimer formation of grancalcin and sorcin seems to be calcium independent. Except for the well-established function of the calpain large and small chain interaction, which together

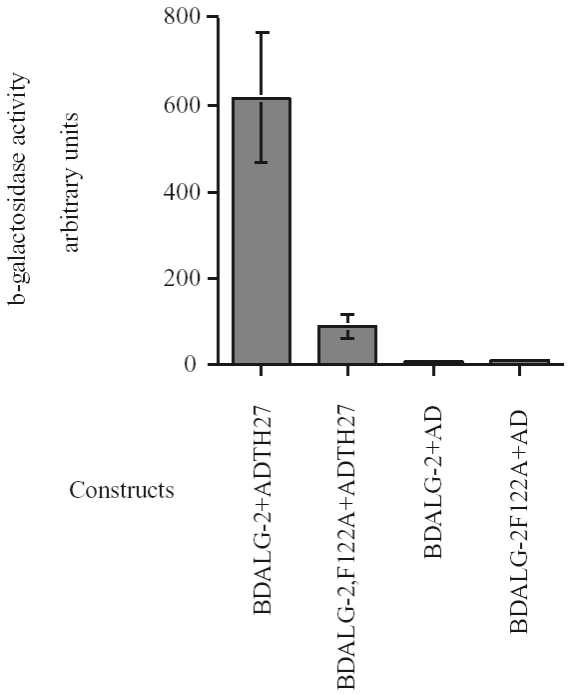

Figure 2. Phe[122] of ALG-2 is crucial for AIP1-binding. Two-hybrid analysis of interaction between the Phe[122]→ Ala ALG2 mutant protein and AIP1 (TH27 fragment). The beta-galactosidase activity in the yeast extracts was determined for four independent clones from each transformation. Values (mean± SD) are given in arbitrary units

form an active protease, the biological significance of the other heterodimeric PEF complexes is not yet known. Heterodimerization may represent a mechanism to fine-tune transmission of a calcium signal by PEF proteins inside the cell via differential target interactions.

As the yeast two-hybrid approach proved to be a powerful tool to study protein-protein interactions among PEF proteins, it was therefore also applied to analyze structural requirements for the ALG-2/target protein interactions. We have shown that the short isoform of ALG-2, ALG-2.1, does not bind the ALG-2 interacting protein AIP1/Alix (20). Lack of interaction between ALG-2.1 and another ALG-2 target, ASK1, was also reported recently (29) (see also chapter 8.2).

By using site-directed mutagenesis, we have demonstrated that Phe[122], one of the amino acid residues lacking in the ALG-2.1 isoform, is required for efficient interaction with AIP1/Alix (Hansen, unpublished results, Figure 2). Interestingly, an intact N-terminus of ALG-2 was also found to be important for this interaction (20). As predicted by the 3D structure of ALG-2, Phe[122] interacts with the N-terminal peptide (Figure 1A). Therefore, Phe[122] may either function in establishment of the structural integrity necessary for correct protein folding or, together with the N-terminal peptide, Phe[122] may be a part of the target protein interaction interface.

7. UBIQUITOUS EXPRESSION OF ALG-2 AND CA^{2+}-DEPENDENT SUBCELLULAR LOCALISATION

ALG-2 tissue distribution has been investigated both, at the level of RNA and the expressed protein. ALG-2 mRNA was found in all mouse tissues examined, i.e., thymus, heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, with the strongest signal in thymus and liver (4). In all those tissues both ALG-2 and ALG-2.1 transcripts were present at the molar ratio of 2:1, respectively (10, Tarabykina, unpublished). Both ALG-2 isoforms are present in the human, mouse, chicken, frog and rat EST databases. The ALG-2 protein expression level in different mouse tissues was recently investigated (30) using the rabbit polyclonal antibody that does not discriminate between ALG-2 and ALG-2.1 (described in Table 1). ALG-2 was detected in all 15 tissues tested (pancreas, uterus, thyroid gland, thymus, testis, submaxillary gland, spleen, prostate, bladder, brain, eye, heart, kidney, liver, and lung), with the highest level in uterus, spleen, and kidney, and the lowest level in the heart, thyroid gland, and pancreas. Furthermore, we have observed ALG-2 expression in a variety of cell lines originating from chicken, hamster, human, monkey, mouse, and rat. Based on these observations we conclude that ALG-2 expression is ubiquitous.

7.1. Antibodies against ALG-2

At present three commercial affinity-purified antibodies against ALG-2 are available from two different vendors (Table 1). We have tested these ALG-2 antibodies in immunoblots in comparison with our affinity-purified rabbit polyclonal antibodies described in (30). ALG-2 was not detected in a human T cell leukemia cell line Jurkat using the commercially available affinity-purified antibodies under conditions recommended by the vendor. In the immunoblots the two goat polyclonal antibodies from Santa Cruz Biotechnologies recognized proteins with molecular weights far from the expected 22 kD of ALG-2, even at the highest tested concentration of the antibodies (30). The Becton Dickinson Transduction Laboratories monoclonal clone 22 antibody against ALG-2 recognized a protein that appeared slightly larger than the band recognized by our polyclonal antibodies on the immunoblots (Table 1). This protein of 23 kD was reported to be degraded upon apoptosis induced by CD95 receptor cross-linking in Jurkat cells (31). Indeed, using the clone 22 ALG-2 antibody we reproduced these results and identified the clone 22 antigen as the co-chaperone protein p23, which is unrelated to ALG-2 (32). Our rabbit polyclonal ALG-2 antibodies failed to detect the cleavage and degradation of ALG-2 under these conditions (32). Based on these observations it can be excluded that the clone 22 ALG-2 antibody and our polyclonal ALG-2 antibodies recognize the same protein.

Taken together these observations let us conclude that neither the ALG-2 antibodies from Becton Dickinson Transduction Laboratories nor from Santa Cruz Biotechnologies are able to detect *bona fide* ALG-2. This

Table 1. Antibodies against ALG-2

Sources, antibody identification	ALG-2 immunogen	Purity, host, subtype	Reactivity, recommended usage	Major bands, immunoblot	Literature reporting use of antibody
SCB, H-14	peptide, N-terminal	affinity purified goat polyclonal	human, mouse, rat IB, IHC	106 kD	30
SCB, A-17	peptide, internal	affinity purified goat polyclonal	human, mouse, rat IB, IHC	74 kD	30
BDTL, clone 22	peptide, mouse AA 162-181	mouse, IgG1, monoclonal	human, mouse, rat, dog IB, IF, IHC	23 kD	13, 14, 30-32, 84, 85, 86
OL anti-recombinant-ALG-2	recombinant full length murine ALG-2	affinity purified rabbit polyclonal	human, mouse, rat, chicken, hamster, monkey IB, IF, IHC, IP	22 kD	30, 32
OL anti-peptide-ALG-2	peptide, mouse AA 117-133	affinity purified rabbit polyclonal	mouse, human IB	22 kD	30

Data in the first four columns are based on the information stated in the product specification sheets, SCB: Santa Cruz Biotechnologies; BDTL: Becton Dickinson Transduction Laboratories; AA: amino acid(s); OL: produced in our laboratory (serum is available from SWANT, Switzerland); IB: immunoblot; IF: immunofluorescence; IHC: immunohistochemistry; IP: immunoprecipitation

implies that the conclusions from papers describing properties of ALG-2 based on the use of these antibodies should be considered with great caution (32) (Table 1).

7.2. Ca^{2+}-dependent translocation of ALG-2 and other PEF proteins

Sub-cellular localization of ALG-2 and several other PEF proteins has been investigated by cell fractionation in a density gradient followed by immunoblotting (26, 33-35). To analyze the influence of calcium on the protein distribution, cell lysis was performed in the presence of Ca^{2+} or EGTA/EDTA. Using this technique PEF proteins have been found to be associated with membranes in the presence of Ca^{2+} and in the cytosol following Ca^{2+} chelation. In rabbit cardiac muscle cells, sorcin was found to be re-distributed from the cytoplasm to the sarcoplasmic reticulum when Ca^{2+} was present in the lysis buffer, whereas in the presence of EGTA sorcin was detected in the cytoplasmic fraction only (33). A similar observation was reported for grancalcin. In the presence of calcium the protein was associated with granules and plasma membrane fractions of human neutrophils, whereas in the presence of EDTA it was detected in the cytoplasmic fraction (34). Calpain from human platelets was detected in the intracellular membranes in the presence of Ca^{2+}, and in the cytoplasm in the presence of EDTA (35). ALG-2 and peflin were detected in the cytoplasm of Jurkat cells in the absence of Ca^{2+}, whereas both proteins were found in the nucleus when the cells were lysed in a Ca^{2+}-containing buffer (26).

Detection of the PEF proteins in sub-cellular fractions obtained by density gradient centrifugation may not reflect the physiologically relevant localization of the proteins as the cell lysis conditions can greatly influence the result. Immunostaining of intact cells is an alternative technique to *in vitro* analysis. Using a polyclonal ALG-2 antibody for immunocytostaining we have observed that ALG-2 is localized to the nucleus but also at vesicle-like structures throughout the cell as well as in the plasma membrane ruffles of several cultured cell lines (Jurkat, NIH-3T3, primary rat astrocytes, HEK-293) (Mollerup and la Cour, unpublished). Elevation of the intracellular Ca^{2+} concentration by ionomycin led to increased ALG-2 immunoreactivity in the nucleus, and also to what appeared to be accumulation of ALG-2 in the vesicle-like structures. The accumulation of ALG-2 in the vesicle-like structures was also observed by live cell fluorescence microscopy of cells transiently transfected with EGFP-ALG-2 (Mollerup, unpublished). These results indicate that Ca^{2+}-binding to ALG-2 can target this protein to different compartments within the cell to fulfill specific functions depending on the external stimuli such as hormones or cytokines known to induce Ca^{2+}-transients within the cell.

8. POSSIBLE FUNCTIONS OF ALG-2

So far molecular pathways involving ALG-2 as a signaling molecule have not been identified. However several putative target proteins have been found using a variety of methods (see Table 2). Even though the biological significance of these interactions has not been shown in most cases, it can be assumed that ALG-2 interaction modifies the known functions of these proteins possibly in a Ca^{2+}- dependent way.

8.1. Apoptosis

Vito *et al*. (4) have generated several 3DO T-cell clones expressing ALG-2 antisense RNA. One of them (ALG-2.clone 1), exhibiting the lowest ALG-2 protein expression level, was protected from Fas, staurosporin, dexamethasone, actinomycin D and C2-ceramide induced cell death. However, other clones with higher remaining ALG-2 expression were not resistant to the latter two stimuli. In addition, it was shown that T-cell receptor driven IL-2 secretion and Fas ligand up-regulation were intact in cells with low ALG-2 expression. This initial work was extended by the same group aiming to define the role of ALG-2 on a molecular level (36). Analysis of the ALG-2 depleted clones showed that caspases were normally activated following TCR and Fas engagement. In addition, over-expression of ALG-2 in fibroblasts induced apoptosis

Table 2. Properties of ALG-2 binding proteins

ALG-2 target proteins	Yeast 2-hybrid	IP (endogeneous)	*In vitro*	Function	Source
AIP1/Alix	+	+	+	Apoptosis, Proliferation, Endocytosis	17, 18
ASK1		+	+	Apoptosis, Junk pathway	29
Fas	+	+	+	Apoptosis, Death receptor signaling	31
Annexin VII	+		+	Endocytosis, Exocytosis, Membrane fusion, Vesicle trafficking, Channel formation	56; Hansen, unpublished
Annexin XI	+		+		55,56
HEED	+			Anterior-Posterior patterning	67
Peflin	+		+	?	26
ALG-2,1	+			?	10

after treatment with ionomycin and phorbolester in combination but not alone (37). Taken together, these results indicate that ALG-2 acts downstream of the apoptosis executing proteases where several pathways converge. It is also possible that ALG-2 functions independently of caspases in alternative pathways leading to apoptosis (38).

Except for the initial studies described above, which all were mostly carried out on 3DO T cells with reduced ALG-2 levels by the group of D'Adamio, only 2 more recent articles by other groups are available supporting the pro-apoptotic function of ALG-2 (39, 40). Rao *et al*. (40) reported that ALG-2 is a mediator of the ER stress-induced cell death pathway based on the observation that, on one hand, immunodepletion of ALG-2 from microsomes reduced the cleavage of caspase 9 after thapsigargin treatment and, on the other hand, siRNA targeted against ALG-2 blocked specifically thapsigargin mediated but not brefeldin-induced cell death.

Several laboratories have attempted to elucidate the function of ALG-2 through identification and functional analysis of target proteins which might be members of signaling pathways regulated by ALG-2. The first identified ALG-2 binding protein was an SH3-binding domain containing protein named AIP1 (17) or Alix (18) for the mouse form, XP95 (41) for the Xenopus laevis form and HP95 (42) for the human homolog. Since among other proposed functions (see below) this protein has been assumed to play a role in apoptosis, its structural and some of its functional properties are discussed in this section in some detail.

AIP1/Alix was independently cloned from liver and brain cDNA libraries using the yeast two hybrid screening technique with ALG-2 as a bait (17,18). A cDNA encoding approximately 400 amino acid residues of the C-terminal region of a novel protein, AIP1/Alix was identified. Vito *et al*. (17) reported that transfection of COS and HeLa cells with the cloned cDNA (clone TH28) partially protected the cells from apoptosis induced by staurosporine and etoposide. Co-transfection with ALG-2 restored the sensitivity to these drugs completely. This may indicate that TH28 sequesters ALG-2 and thereby inhibits its pro-apoptotic functioning mediated by interaction with AIP1/Alix. Over-expression of full length AIP1/Alix in post-mitotic cerebellar neurons induced caspase activation after K^+ deprivation, while over-expression of the C-terminal part of AIP1/Alix blocked caspase activation (39). In the same study the authors found that deletion of a stretch of 12 amino acid residues containing PXY repeats within the C-terminal proline-rich domain of AIP1/Alix, which is necessary for ALG-2 binding (see Figure 3), abolished the effect of full length AIP1/Alix expression. These results indicate that ALG-2 in a complex with AIP1/Alix and possibly other proteins promotes neuronal cell death.

Since the ALG-2 interaction with AIP1/Alix was found to be strictly Ca^{2+}-dependent *in vitro* (18) it is likely that ALG-2 and AIP1/Alix can only interact when cells experience elevated Ca^{2+} levels. Using the yeast two-hybrid system we have shown that the efficiency of the ALG-2/AIP1/Alix interaction is much lower than the efficiency of the ALG2/ALG2.1 heterodimerization (10). Considering that the ALG-2.1 isoform was not able to interact with AIP1/Alix under the conditions of the experiment we speculate that ALG-2.1 possibly can regulate the availability of ALG-2 for AIP1/Alix interaction and thereby the AIP1/Alix function.

The *S. cerevisiae* AIP1/Alix homologue Bro-1 contains a region (named Bro-1 like domain) (43), which is also present in AIP1/Alix, XP95 and HP95 (44) (Figure 3). The Bro1-like domain is also found in other proteins, which do not seem to be orthologs of Bro1, e.g., in the human rhophilin-1 and rhophilin-2 (45) and in the human tyrosine phosphatase candidate tumor suppressor HD-PTP (46). Besides the highly conserved N-terminal Bro1-like domain, AIP1/Alix/XP95/HP95 also share homology within the C-terminal proline-rich sequence known to be the ALG-2 docking site (17, 18) with several SH3-binding (PXXP) domains. In addition, AIP1/Alix/XP95/HP95 also contains a conserved src-kinase phosphorylation site that is flanked by two FxDxF motifs, known to be able to bind the AP-2 clathrin adaptor protein (47).

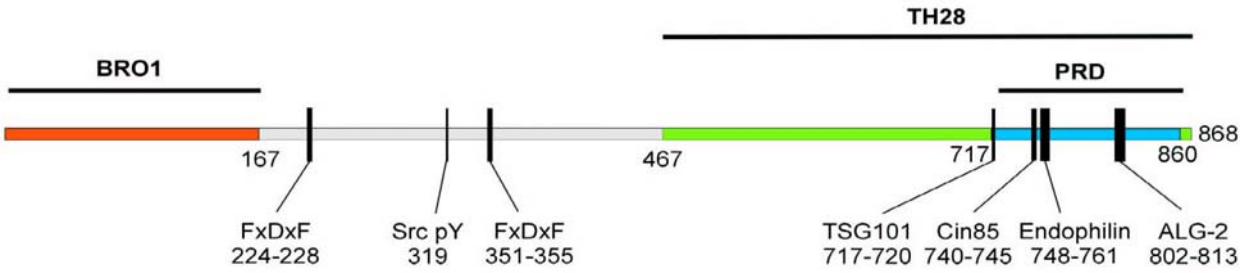

Figure 3. Human AIP1/Alix domain structure. AIP1/Alix contains a BRO1 domain with unknown function, and a proline rich domain (PRD) with several SH3 binding domains. The FxDxF motifs (47) are localised on both sides of the src kinase phosphorylation site (KKDNDFIpYH) that was verified in the *Xenopus laevis* XP95 homolog (41). The consensus phosphorylation site is conserved among the AIP1/Alix homologs. TSG101 binding site (PSAP) (65) Cin85 binding site (PTPAPR) (88). Endophilin binding site (62). ALG-2 binding site (39).

Bro1 has been shown to be required for general amino acid permease Gap1 down-regulation as well as for the direct vacuolar sorting of Gap1 and for the ubiquitination and stress-induced down-regulation of the uracil permease Fur4 in *S. cerevisiae* (48). Bro1 was found to be a cytoplasmic protein associated with the endosomal compartment in *S. cerevisiae*, where it plays a crucial role in the formation of the late endosomal compartments containing luminal vesicles (44).

AIP1/Alix has been found to interact with rat SETA (*S*H3 domain containing protein *e*xpressed in *t*umorigenic *a*strocytes) by the yeast two-hybrid analysis and pull-down with SETA-GST (91). Independently, the same protein named Ruk reviewed in (49), was identified, as a negative regulator of the PI 3-kinase (50). Over-expression of Ruk in cultured primary neurons induced apoptosis. This effect, however, could be overruled by co-expression of a constitutively active form of p110gamma or the downstream effector PKB/Akt (50). The human homolog of SETA/Ruk is CIN85. CIN85 protein mono-ubiquitination was shown to be involved in the ligand-induced degradation of EGF receptors (51) and hepatocyte growth factor receptor Met (52) following receptor ligand activation.

Stable over-expression of the human AIP1/Alix homolog HP95 in HeLa cells revealed multiple effects including: (a) induction of G1-phase arrest, (b) promotion of detachment-induced apoptosis (anoikis), (c) inhibition of detachment of viable cells from the substratum, and (d) reduction of tumorigenicity (42). In non-transformed NIH3T3 cells over-expression of HP95 promoted flat cell morphology and slowed down the proliferation rate of the cells (42). HP95 down-regulation, on the other hand, led to increased proliferation. Therefore, HP95 seems to have the characteristics of a protein that is pro-apoptotic and acts as a negative regulator of cell transformation.

Another possible link of ALG-2 with apoptotic processes could be through its postulated interaction with the cytoplasmic domain of the Fas receptor. ALG-2/Fas interaction has been shown using the yeast two-hybrid assay as well as an *in vitro* technique by GST pull-down assays (31). The specificity of the two-hybrid interaction was shown by using a Fas death domain construct with a point mutation fused to the DNA binding domain. *In vitro* interaction using GST-ALG-2 and ^{35}S labeled Fas was demonstrated to be Ca^{2+}-dependent. It was also postulated that ALG-2 is cleaved upon apoptosis induction into a 19 kD product, which translocates from the plasma membrane to the cytosol during Fas-mediated apoptosis. It has to be mentioned that Western blot analysis and immunocytochemistry were done with an ALG-2 antibody, which clearly does not recognize ALG-2 as reported (30, 32) and discussed in chapter 7.1.

8.2. Proliferation

Using a specific ALG-2 antibody in immonohistochemical studies La Cour *et al*. (30) found that ALG-2 is over 3-fold up-regulated in rat Morris hepatoma tissue as compared to normal liver tissue indicating a proliferative role of ALG-2. Moreover, ALG-2 was found to be significantly up-regulated in large cell, small cell, and squamous cell lung carcinomas as well as in lung adenocarcinomas from 263 patients. Analysis of commercial tissue arrays of several tumor types including colon carcinomas, bladder tumors and hepatocellular carcinomas supports the view that ALG-2 might be generally up-regulated in rapidly proliferating tissues. Krebs *et al*. (13, 14) came to the conclusion that ALG-2 is generally up-regulated in tumors and is most abundant in metastatic tissue. However, we found that the ALG-2 expression levels in 4 different types of lung metastasis are similar or lower than in the corresponding primary tumors (30). Since the two studies by Krebs *et al*. (13, 14) were done using a commercial antibody which does not recognize ALG-2, but the p23 protein (32), we do not further discuss this discrepancy.

Using rapid analysis of gene expression (RAGE) for identification of genes whose expression is controlled by E2F1, Wang *et al*. (53) identified a novel gene, named EIG-2, that was up to 5-fold induced in cells expressing E2F1. DNA database analysis showed that EIG-2 is identical to AIP1/Alix (13). Another putative ALG-2 target, HEED (see also chapter 8.4), was also found among E2F1 inducible genes (Christian Helin, personal communication). Since at least two E2F-dependent ALG-2 targets are expected to be up-regulated in the G1/S transition phase of

the cell cycle, these ALG-2/target protein complexes may play a role in cell proliferation.

The ALG-2 interacting protein ASK-1 is a MAPKKK acting upstream of JNK upon induction of apoptosis with e.g. Fas, TNF, oxidative stress, DNA damage and polyglutamine repeats (54). Hwang *et al*. (29) have shown that ALG-2 binding occurs to the C-terminal part of ASK1 in BOSC23 (a retrovirus packaging cell line) as well as *in vitro*. *In vitro*, the interaction seems to be Ca^{2+} independent. Similar to AIP1/Alix, ASK-1 does not interact with the ALG-2,1 isoform, while it is able to bind a truncated ALG-2 (ALG-2-23N) (29). This indicates that structural requirements for the ALG-2/ASK-1 complex formation are similar to those of ALG-2/AIP1/Alix. Co-transfection of ALG-2 and ASK1 shifts the localization of ASK1 from the cytoplasm to the nucleus. Since ASK1 activates JNK only when localized in the cytoplasm, further phosphorylation of c-Jun by JNK is reduced. These data indicate that ALG-2 could be a survival factor acting in an anti-apoptotic fashion in the JNK pathway.

ALG-2 was shown to bind annexin VII and annexin XI *in vitro* (55,56), the proteins which have been proposed to function in a multitude of cellular activities such as membrane fusion and membrane channel formation (see references in 56). Similar findings were made in our lab using the yeast two-hybrid system (Hansen, unpublished). Recently, it was found that annexin VII binds galectin-3 (57). This multifunctional oncoprotein has been shown to protect cells from undergoing apoptosis by stabilizing mitochondrial integrity. Galectin-3 translocates during apoptosis to perinuclear mitochondrial membranes where it counteracts cytochrome C release. Annexin VII interferes with this process and thereby acts in a pro-apoptotic fashion. A possible explanation for the anti-apoptotic function of ALG-2 could therefore be at the level of abolishing the function of annexin VII. However, it could as well be argued that the ALG-2/annexin VII interaction promotes the annexin VII function thereby acting as a pro-apototic protein (see also chapter 8.1)

8.3. Exocytosis/endocytosis/protein trafficking

Interestingly, two targets of ALG-2, annexin VII and AIP1/Alix, and also the annexin VII binding protein galectin-3 were among 21 newly identified proteins found to be enriched and which co-localized in the dendritic cell-derived exosomes, which are distinct from apoptotic vesicles (58). The presence of these and other proteins known to be related to apoptosis indicate that there may be structural and/or functional relationships between apoptotic and endocytotic pathways. Likewise, AIP1/Alix and galectin-3 were found to be enriched in phagosomes shed from macrophages (59). It has been speculated that phagolysosomes may induce apoptosis in macrophages by an unknown mechanism involving the above-mentioned molecules (60).

Since Bro1/AIP1/Alix could function to regulate trafficking in the endosomal compartments and multivesicular bodies (see also chapter 8.1), and since exosome release is a Ca^{2+}-dependent process (61), the Ca^{2+}-dependent ALG-2/AIP1/Alix interaction may play a regulatory role in this scenario. We have observed that part of the endogenous ALG-2 and AIP1/Alix immunoreactivity as well as EGFP-tagged ALG-2 and AIP1/Alix are localized to small vesicular structures and to ruffles at the plasma membrane (Mollerup, unpublished, see also chapter 7.2). These sites may be the place of an AIP1/Alix/ALG-2 interaction, if present *in vivo*, to occur.

AIP1/Alix was found to interact with endophilins (SH3p4, SH3p8 and SH3p12) through a 14 amino acid residues motif which includes an SH3-binding domain in the C-terminal part of AIP1/Alix (Figure 3). A similar motif is present in two other endophilin binding proteins, synaptojanin 1 and germinal center kinase-like kinase (62). Transient over-expression of the C-terminal part of AIP1/Alix led to cytoplasmic vacuolization into perinuclear tubulo-vesicular structures. The co-expression of endophilin 1 and the C-terminal part of AIP1/Alix resulted in the appearance of few but very large vacuoles (62).

Recent data from several laboratories indicate that AIP1/Alix is involved in the trafficking of vesicles in the endosomal compartments. AIP1/Alix was shown to interact with proteins of the endosomal sorting complex required for transport (ESCRT), type I and III (63-66). The ESCRT complexes are needed for the sorting of vesicles with ubiquinated membrane proteins into multivesicular bodies. The human immunodeficiency virus (HIV) Gag p6 and the equine infectious anemia virus (EIAV) Gag p9 proteins were found to specifically interact with AIP1/Alix in the course of virus budding at the plasma membrane. It was hypothesized that the virus proteins via AIP1/Alix recruit the ESCRT components of the cell, and thereby enable the virus budding process at the cell surface, analogous to vesicle budding into the multivesicular bodies

A further possible link of ALG-2/AIP1/Alix signaling in endocytosis comes from the association of AIP1/Alix with CIN85, a protein which has been shown to be involved in c-MET and EGF receptor down-regulation by endocytosis in a complex with Cbl and endophilin (51, 52). Based on these findings it can be speculated that AIP1/Alix promotes down-regulation of receptor tyrosine kinases. Since ALG-2 has been found to be up-regulated in cancer tissue (30), the protein could therefore be a negative regulator of AIP1/Alix, inhibiting its anti-proliferative effects.

8.4. Development

Lee *et al*. (67) found that ALG-2 interacts with the C-terminal region of HEED (Human Embryonic Ectoderm Development) using a yeast two-hybrid system. HEED is the human homolog of mouse EED and the *Drosophila* protein ESC which has been shown to be a transcriptional silencer of homeotic genes responsible for the anterior-posterior patterning during embryogenesis (68). However, the implication of ALG-2 in development has not been investigated so far.

9. ALG-2 IS EVOLUTIONARILY CONSERVED

Homologs of ALG-2 have been found in a wide range of species extending from mammals to insects and

plants. Aligning protein sequences from various organisms reveals an unusually high degree of conservation. Human, mouse and rat ALG-2 differ in only one out of 191 amino acid residues, whereas chicken ALG-2 shares 90% identity with the human protein, fruit fly – 68%, thale cress – 33% and slime mold only 26%. All the ALG-2 proteins have five EF-hands. These domains are highly homologous among the proteins from different species. The C-terminal residue of the majority of the ALG-2 homologs is situated immediately after their predicted fifth EF-hand, the exception being the extended C-terminal region of the *Drosophila melanogaster* (fruitfly) homolog. The most variant part among all the proteins is the N-terminal region, which ranges in length from 164 residues in *Arabidopsis thaliana* (thale cress) to just four residues in *Caenorhabditis elegans* (nematode). A general feature of the N-terminal sequences is that they are rich in proline, alanine, tyrosine and hydrophobic amino acid residues. The SignalP prediction server (http://www.cbs.dtu.dk/) assigns a chloroplast localization signal to part of the long N-terminus of the *A. thaliana* ALG-2, but does not predict any function or sub-cellular localization of the N-terminal regions of any other ALG-2 protein (69). The mammalian ALG-2 homologs have glycine and proline rich N-termini of equal length, which for the mouse protein has been predicted to fold back into a hydrophobic pocket (20) (see chapter 4). Since this sequence has similarities to ALG-2 recognition sequences in AIP1/Alix and annexin VII, it can be speculated that it may interfere with ALG-2 target interaction and could thereby regulate the activity of ALG-2. The amino acid residues forming this putative hydrophobic pocket for harboring the N-terminal sequence are conserved among vertebrates irrespective of the glycine/proline content and length of their N-terminal regions. One of the hydrophobic residues lining the pocket in mouse is Phe^{122}, which is not present in the short splice variant (see also chapter 4).

In addition to coding sequences, also particular genomic sequences are highly conserved. One example is the above-mentioned conservation of an alternative splice site. The positions of the five introns with respect to the coding sequences are conserved between the human, mouse, rat and chicken ALG-2 gene sequences. In human the ALG-2 gene is located on chromosome 5p15.2-pter, in mouse on chromosome 13C1, in rat on chromosome 1p11 and in chicken on chromosome 2q21-23 (data from NCBI and our unpublished results). The positions of ALG-2 on the human, mouse and rat chromosomes are conserved as follows the synteny between these organisms (NCBI and (70)). Interestingly, in general the organization of the human genome has been found to be more closely related to that of chicken than mouse, despite a modal chromosome number of 78 in chicken (71). Many genes found on human chromosome 5p have homologs on the chicken sex chromosome Z. However, chicken ALG-2 is located on chromosome 2 indicating no evidence of conserved synteny in this case. Common variable immunodeficiency is one of the most frequent immunodeficiencies in humans and has recently been linked to heterogeneity of chromosome 5p. However, heterogeneity in the exons of the ALG-2 gene was not found (72).

A phylogenetic analysis of ALG-2 homologs (Figure 4) indicates early speciation from a single ancestral gene, as the tree topology does not change significantly with different calculation methods. The ALG-2 tree conforms to the accepted version of eukaryote evolution, allowing for the uncertainty of the branching of the dictyostelid lineage (73). The two ALG-2 homologs of the slime mold *D. discoideum* are the only homologs, apart from the mouse protein, to have been characterized in detail (74). Especially the Dd-ALG-2a homolog shares many features with mouse ALG-2: upon homology modeling their structures are similar and binding of Ca^{2+} exposes hydrophobic residues. As measured by surface plasmon resonance (74) Dd-ALG-2a homodimerizes and can interact with both murine and dictyostelid AIP1/Alix (Aubry, Mattei and Klein, personal communication). The Dd-ALG-2b homolog resembles the short form of ALG-2, ALG-2.1, as it neither interacts with murine nor dictyostelid AIP1/Alix. However, no ALG-2b homodimers were found and heterodimerization only occurred when ALG-2b was the immobilized partner (74).

10. ALG-2 GENE DISRUPTION REVEALS NO PHENOTYPES

Homologous recombination as well as more recently developed techniques such as RNA interference, anti-sense RNA, and siRNA have been employed to reduce expression of ALG-2. Several of these approaches have been used by different groups working on diverse models in the attempt to elucidate the function of ALG-2.

The first published result on the disruption of ALG-2 came as part of the reverse genetic screen and functional analysis of the nematode *Caenorhabditis elegans* chromosome I by RNA interference (75). Several important apoptosis related genes were first described in this organism. The predicted *C. elegans* ALG-2 homolog is a putative calcium binding protein and the gene encoding it is comprised of 4 exons and resides on chromosome I. Feeding nematodes with bacteria expressing double stranded ALG-2 RNA did not result in any discernable phenotype (75, 76). During this screen approximately 87% of the 2769 predicted genes on chromosome I were targeted. These results could be due to either a subtle/conditional phenotype or due to ineffective RNAi, neither of which was scored during the screen (75).

In the cellular slime mold *Dictyostelium discoideum* two ALG-2 homologs, Dd-ALG-2a and Dd-ALG-2b, have been characterized (see also chapter 9) (74, 77). Both Dd-ALG-2a and -2b are expressed constitutively during all developmental phases of *D. discoideum*. Despite the fact that sporulation of *D. discoideum* requires apoptosis during stalk formation, only few dictyostelid proteins have been found to be homologous to proteins involved in apoptosis in mammals. Apart from the two ALG-2 homologs only AIF and two putative paracaspases have been found. Nevertheless, disruption by homologous

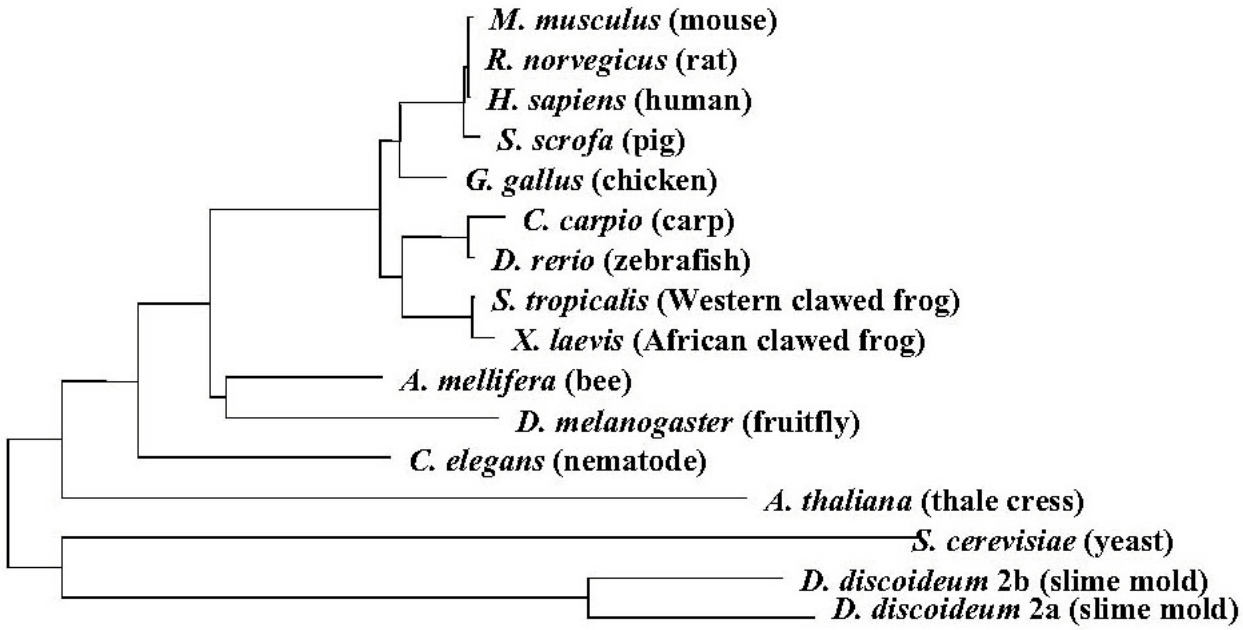

Figure 4. ALG-2 dendrogram. Phylogenetic tree of ALG-2 homologs (generated by TreeCon 1.3b (89) upon alignment of proteins using the Clustal X1.81 program (90)). The protein sequences are derived from or were translated from the following files: *Apis mellifera* - honey bee (BI508387), *Arabidopsis thaliana* - thale cress (AAL32576), *Cyprinus carpio* - carp (CA969814), *Caenorhabditis elegans* - nematode (NP491447), *Dictyostelium discoideum* - slime mold (2a: AF358911; 2b: AF358912), *Drosophila melanogaster* – fruitfly (AAF45393), *Danio rerio* – zebrafish (CA471501), *Gallus gallus* - chicken (BI067670), *Homo sapiens* - human (AAC27697), *Mus musculus* - mouse (NM011051), *Rattus norvegicus* - rat (CA338645), *Saccharomyces cerevisiae* - budding yeast (NP011572), *Sus scrofa* - pig (CB483177), *Silurana tropicalis* - Western clawed frog (BQ397553), *Xenopus laevis* (AW460954) - African clawed frog

recombination of either Dd-ALG-2a or Dd-ALG-2b or both in the same clone had no effect on the development and differentiation of *D. discoideum* (74). During the search for the ALG-2 homologs in *D. discoideum*, an AIP1/Alix homolog was found which interacts with Dd-ALG-2a, but not with Dd-ALG-2b. The AIP1/Alix-null mutants had no phenotype at the unicellular stage of the slime mold, but display a severe defect in the formation of the stalk carrying the sporangium in *D. discoideum* during the multicellular stage (Aubry, Mattei and Klein, personal communication).

Almost simultaneously with the report on ALG-2 gene disruption in *D. discoideum* (74), Jang *et al*. published their findings on ALG-2 deficient mice (78). The mice were generated from ES cells carrying a disruption of exon I of the mouse ALG-2 gene, which completely abolishes ALG-2 protein expression in homozygous ALG-2 -/- mice. The mice were healthy and fertile, indicating that ALG-2 is not required for the development and survival of mice. An examination of both the T and B-cell populations in the ALG-2 deficient mice showed that these were not different from the populations found in wild type mice. Likewise, T cells from the ALG-2 deficient mice functioned normally as seen by their proliferative and IL-2 productive response to CD3 stimulation. Originally, ALG-2 was described as being required for T-cell receptor induced apoptosis in 3DO T-cell hybridomas (4). However, the ability of both mature, activated T cells and thymocytes from the ALG-2 -/- mouse to undergo apoptosis upon stimulation of the TCR was comparable with that of wild type T cells and thymocytes, as was apoptotic stimulation of ALG-2 -/- thymocytes with anti-Fas antibody and dexamethasone. Despite further examinations, no discernable phenotypes were found (78).

The original ALG-2 experiments were performed in 3DO mouse T-cell hybridomas using a partial ALG-2 clone in the antisense orientation to down-regulate the expression of the ALG-2 protein (4). Apart from being immortalized upon culturing, 3DO cells differ from wild type T cells in that they (as thymocytes) undergo apoptosis upon death receptor stimulation as well as T-cell receptor stimulation, while freshly isolated, non-activated T cells do not. (Vito, personal communication). Between 15% and 30% of the 3DO cells with down-regulated ALG-2 expression died following dexamethasone treatment, T cell or Fas receptor stimulation compared to 60-90% of the "wt" 3DO cells (4). However, equal amounts of ALG-2 -/- thymocytes compared to wild type thymocytes died following the same treatments. Furthermore, activated ALG-2 -/- and wild type lymphocytes die at the same rate upon TCR stimulation (78). The discrepancies between the 3DO and ALG-2 -/- cell experiments could perhaps be explained by differences in the experimental set-ups as well as by biological differences between the established cell line and primary lymphocytes which represent a functionally heterologous population. Functional redundancy between the penta-EF-hand proteins may be the reason why disruptions of individual PEF family members in mice give no major phenotypes. Grancalcin -/- mice, as the ALG-2 -/-

mice, show no readily visible phenotype (79). The cysteine proteases, calpains, are the only members of the PEF family for which the function has been clearly elucidated (reviewed in (27)). Still, disruption of one of the two forms of the catalytic subunit has no major phenotype in mice, whereas disruption of the regulatory subunit is embryonically lethal (80, 81). As the PEF proteins are very similar in their primary structure, dimerization and mode of intracellular translocation (12), they could be functionally redundant per se, or the lack of phenotype in the knockout mice could reflect an induced redundancy. Induced redundancy, where one protein substitutes the function of another only upon the prolonged absence of the first protein, could possibly be proven by the use of an inducible knock-out system.

11. PERSPECTIVES

Calcium homeostasis is required for the correct functioning of all eukaryotic cells. Loss or disturbance of calcium regulation can lead to initiation of the "physiological" cell death program called apoptosis (5). On the other hand subtle changes in calcium distribution within a cell can also be utilized to counteract cellular death programs. Besides this dual role of calcium with respect to cell survival and cell death, calcium regulates a variety of other cellular processes making biochemical dissecting of signaling pathways difficult. Several molecules involved in calcium handling and signaling such as calcium-dependent kinases, phosphatases, calcium pumps and exchangers as well as calcium binding and storage proteins of the modulating and buffering type have been implicated in apoptotic processes.

ALG-2 is the only so far known calcium-binding protein discovered in a functional screen for apoptotic proteins. After the first report on ALG-2 in 1996 (4) only two studies, both published in 2004 (39, 40), indicate that this protein plays a role in apoptosis. However, molecular knowledge on ALG-2 dependent signaling pathways is still obscure. On the other hand some of the data available on ALG-2 point to its role rather in cell proliferation or survival (29, 30). The list of putative ALG-2 targets continues to grow, which may indicate that this protein is truly multifunctional as known for calmodulin. The following properties support this view: as calmodulin, ALG-2 is highly conserved in contrast to e.g. calcium buffering proteins and it is expressed ubiquitously. So far all cell lines and tissues analyzed express ALG-2. ALG-2 binds calcium in the μM range and undergoes a conformational change upon calcium binding. Several proteins have been shown to be pro- and anti-apoptotic or pro- and anti-proliferative depending on the circumstances. These include e.g. c-Myc and p21Cip. The latter example is interesting since present in the nucleus this protein inhibits cell cycle and proliferation but if present in the cytoplasm it contributes to the transformed phenotype (82). As ALG-2 localization depends on the intracellular calcium concentration (see chapter 7.2), this protein could have different functions depending on its localization or the balance between opposing activities could be altered.

ALG-2 has the property to form homodimers but also heterodimers with its splice form ALG-2.1 and peflin, two other members of the penta-EF-hand family of proteins. In addition, at high concentration and in the presence of calcium it can polymerize. Whether such complexes are of physiological relevance has to be shown. At any rate homo- and heterodimerization or multimerization are expected to alter the calcium sensitivity and the target recognition properties of ALG-2 and thereby contribute to the proposed multifunctionality of this protein.

ALG-2 seems to be a nonessential gene in several species (*C.elegans* (75), *D. discoideum* (74) and *M. musculus* (78)), loss of which may be compensated by other genes from the same family. The best-known ALG-2 interacting protein, AIP1/Alix has recently gained much attention due to the fact that it is a critical adaptor protein linking various proteins which are needed in endocytosis and virus budding (63-66). Tsg 101, an essential component for multivesicle body formation is a member of the ESCRT-I protein complex and binds directly to AIP1/Alix at its C-terminal region where also ALG-2 binds. Since calcium regulates various aspects in exocytosis and endocytosis (83) it is tempting to speculate that ALG-2 might be a calcium sensor to control virus release.

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