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The role of calcium in apoptosis

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In this chapter various aspects of apoptosis or programmed cell death (PCD) influenced by calcium as a mediator of signal transduction have been reviewed. Attention has been focused on recently described calcium-binding proteins such as ALG-2 or on a new calcium/calmodulin-dependent kinase, the death associated protein kinase or DAP-kinase. Both play a central role in apoptotic processes. Calcineurin, which normally is involved in the regulation of T-cell proliferation, is reported to interact with the apoptosis protection protein bcl-2. Its possible involvement in the decision process whether T-cell activation leads to proliferation or apoptosis is discussed.

Keywords: apoptosis; programmed cell death (PCD); calcium; DAP-Kinase; calcineurin; ALG-2

Introduction

About 25 years ago the term apoptosis was coined (Kerr *et al*. 1972). Apoptosis, or programmed cell death (PCD), is a process during which cells shrink and dissociate from their surrounding neighbours, their organelles retain in size, and in the nucleus chromatin forms dense aggregates on the nuclear membrane and, eventually, undergoes fragmentation. On the other hand, necrotic cells swell, their mitochondria enlarge, the plasma membranes disrupture, but the nuclear changes are marginal.

PCD is an important mechanism during development which has emerged in multicellular organisms to remove unnecessary, damaged or aged cells. Therefore, abnormal resistance towards apoptosis may lead to autoimmune diseases or cancer, whereas uncontrolled enhancement of apoptotic processes could favor chronic pathologies such as neurodegenerative diseases (e.g. dementia of the Alzheimer type) or immune deficiencies such as AIDS.

Investigation of the detailed mechanisms involved in PCD was stimulated in recent years by the accumulating evidence that a number of highly conserved proteins are involved in the complex apoptotic pathways by either causing or preventing cell death. This was mainly due to detailed studies of the nematode *Caenorhabditis elegans* (*C. elegans*) which have revealed the exact lineage and developmental fate of every single cell in this organism. During development, 131 of the total 1090 cells of *C. elegans* are predestined to die. This apoptotic process is controlled by the coordinated expression of the so-called death proteins ced-3, ced-4, and ced-9 (Ellis *et al*. 1991). These proteins of which highly conserved homologues have been identified in higher organisms including mammals have been shown to be of central importance for controlling various pathways of PCD. Loss of function mutations in the corresponding genes prevented the death of specific cells during development of *C. elegans* (Yuan *et al*. 1993; Shaham & Horvitz 1996; Hengartner *et al*. 1992). Therefore these genes were designated as *C. elegans* death (ced) genes. Ced-3 belongs to a steadily growing family of cysteine proteases homologous to interleukin-1β-converting enzyme (ICE) which are now called caspases. Ced-9 was identified as a member of the bcl-2 family which includes both inhibitors as well as inducers of apoptosis (Reed 1997). Recent studies identified also a mammalian

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homologue of ced-4, Apaf-1 (Zou *et al*. 1997) which apparently is involved in the regulation of the other members of the cell death genes due to protein-protein interaction (Chinnaiyan *et al*. 1997; Wu *et al*. 1997), but a detailed understanding of the function is still missing.

The ability to undergo apoptosis is a property which most, if not all, animal cells have. The activation of the intrinsic suicide program can be triggered by a variety of stimuli including DNA fragmentation, growth factor withdrawal, admission of steroid hormones such as glucocorticoids, and calcium influx. In this review I will concentrate on some aspects of the influence of calcium on apoptotic processes which gained much attention in recent years. A more general information concerning a wide variety of different aspects of apoptosis could be obtained from a number of recently published reviews and references cited therein (Thompson 1998; Penninger & Kroemer 1998; Nicotera & Orrenius 1998; McConkey & Orrenius 1997).

Calcium is one of the most versatile second messengers involved in cell growth, differentiation, and also in programmed cell death. It plays its pivotal role through a specific class of proteins, the so-called EF-hand type Ca^{2+}-binding proteins (see Kawasaki *et al.* 1998; Nelson & Chazin 1998), some of which are directly involved in the control of apoptosis linked processes (see below). In resting cells, the concentration of intracellular free Ca^{2+} lies between 50 and 200 nM, whereas the calcium concentration in the extracellular space or in the reticular system within the cell is between 2–5 mM. Small changes of the resulting steep concentration gradient across membranes by releasing Ca^{2+} from intracellular stores or by influx of Ca^{2+} from extracellular media through Ca^{2+}-channels can be used to trigger Ca^{2+}-dependent events. Therefore calcium homeostasis within the cell is tightly controlled by a number of calcium-transporting systems as reviewed by Guerini (1998). On the other hand, already 30 years ago it was pointed out by Fleckenstein, who pioneered the development of Ca^{2+}-channel antagonists, that excessive entry of calcium into cells may be cytotoxic and therefore could lead to cell death (see Fleckenstein 1984). Here we will discuss various aspects under which conditions changes of intracellular calcium may influence apoptosis.

Stimulation by glucocorticoids

Kaiser and Edelman demonstrated more than 20 years ago that glucocorticoid-stimulated apoptosis of thymocytes was associated with an increase of Ca^{2+} influx (Kaiser & Edelman 1977). These observations were later confirmed by McConkey *et al*. (1989). Similar to promoting Ca^{2+} increases in apoptotic cells by glucocorticoids such as dexamethasone calcium influx into cells could also be associated with different forms of radiation induced lymphocyte apoptosis (Spielberg *et al*. 1991). Both events lead to an activation of protein tyrosine kinases (Schieven *et al*. 1993; Yao & Scott 1993) which in turn phosphorylate phospholipase Cγ thereby activating it (Rhee & Choi 1992). Subsequently, activated phospholipase Cγ cleaves phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol-1,4,5-trisphosphate (IP3). The latter binds to the IP3-receptor located in the endoplasmic reticulum (ER) membrane thereby releasing Ca^{2+} through the Ca^{2+} channel associated with the IP3-receptor. In these examples the initial increases of intracellular Ca^{2+} leading to apoptosis occur through controlled physiological mechanisms which are also used in alternative responses such as proliferation.

In this context the question could become interesting how the cell decides on the molecular level between the proliferative or the apoptotic pathway which may be answered by some recent findings. Jayaraman *et al*. (1996) provided evidence that upon T-cell stimulation the IP3-receptor1 (IP3R1) gets specifically activated by tyrosine phosphorylation resulting in IP3-induced calcium release which finally leads to IL-2 production and proliferation of T-cells. On the other hand, the same Laboratory could also show that T-cells lacking IP3R1 due to the presence of antisense RNA fail to increase intracellular Ca^{2+} or to synthesize IL-2 upon T-cell receptor stimulation (Jayaraman *et al*. 1995). These results indicate that the presence of IP3R1 is required for Ca^{2+}-induced IL-2 production in activated T-cells triggering T-cell proliferation. On the other hand, the same Laboratory also provided evidence that if T-cells were deficient in IP3R1 those cells were resistant to apoptosis induced by dexamethasone, T-cell receptor stimulation, ionizing radiation and Fas (Jayaraman & Marks 1997). This block, however, could be overcome by artificially raising cytoplasmic calcium levels (e.g. by using calcium ionophores). In contrast, it was shown recently by Khan *et al*. that in lymphocytes undergoing apoptosis in response to dexamethasone synthesis of IP3R3, but not of IP3R1, is specifically induced and that blocking expression of IP3R3, but not of IP3R1, with antisense oligonucleotides inhibited triggered apoptosis (Khan *et al*. 1996). Even if the results described above are somewhat contro-

versial, they both agree that IP3-dependent Ca^{2+} release is essential, whether stimulation leads to proliferation or apoptosis, but what determines the switch in either direction is probably regulated by other signals.

Next to glucocorticoids there are a number of other stimuli which can induce apoptosis in thymocytes. These include γ-irradiation (see above), stimulation by antibodies against Fas/CD95 antigen (Owen-Schaub *et al.* 1992) and removal of growth factors from the culture medium (Duke & Cohen 1986). Most of these stimuli have in common the rise of intracellular calcium subsequent to the induction of PCD. Further key molecules associated with the regulation of apoptosis are the cysteine protease IL-1β-converting enzyme (ICE; homologue of ced-3 as indicated before) and the tumor suppressor p53, but which apparently belong to different pathways. Thus it has been shown that ICE-deficient mice develop normally, but apoptosis could be induced in their thymocytes only by the application of glucocorticoids or by ionizing radiation, but not by Fas/APO-1 antibodies indicating that ICE is one of the components of the Fas pathway (Kuida *et al.* 1995; Li *et al.* 1995; Los *et al.* 1995). On the other hand, if thymocytes of p53 deficient mice are exposed to γ-irradiation they are resistant to apoptosis, but not if exposed to other stimuli indicating that p53 is essential for apoptotic pathways induced by DNA-damaging agents (Lowe *et al.* 1993).

As already pointed out before one of the consequences of the induction of apoptotic pathways by different stimuli is the increase of $[Ca^{2+}]_i$ indicating that the different stimulating signals leading to apoptosis converge in the elevation of the intracellular free Ca^{2+}-concentration as a general mediator of apoptotic events. This view was corroborated by the recent findings made by Jayaraman & Marks that T-cells lacking IP3R1 (see above) were resistant to apoptosis independent whether in these cells apoptosis was induced by either dexamethasone, Fas/APO-1, γ-irradiation or T-cell receptor stimulation (Jayaraman & Marks 1997). Evidence was provided that T-lymphocytes deficient in expressing IP3R1 due to the presence of antisense RNA lacked IP3-induced intracellular calcium release, even if the other isoforms, IP3R2 and IP3R3, were present. This resistance to apoptosis could, however, be overcome by raising the cytoplasmic Ca^{2+} levels due to the application of calcium ionophores. In addition, it was shown that apoptosis mediated by T-cell receptor stimulation was independent of extracellular calcium, since extracellular calcium influx was not required (Jayaraman & Marks 1997). All these data point to the fact that calcium plays a key role in triggering apoptotic processes induced by a number of stimuli.

As indicated before one of the key components in apoptotic pathways is the cysteine protease ICE. Its importance became especially apparent due to studies with the nematode *C. elegans* which led to the discovery that one of the members in *C. elegans* death proteins, ced-3, was the homologue to ICE (Yuan *et al.* 1993). In a recent study by D'Adamio and his colleagues attention was focused to identify existing links between the role of ICE in apoptotic processes and the recently discovered apoptotic linked genes ALG-2 and ALG-3 (Lacana *et al.* 1997; Vito *et al.* 1996). These authors made the interesting observation that T-cell hybridomas depleted of ALG-2, a Ca^{2+}-binding protein of the EF-hand type family (Vito *et al.* 1996), were protected against PCD induced by a variety of stimuli including dexamethasone and Fas/CD95 triggering. The authors provided evidence that members of the ICE protease family were activated upon stimulation by either Fas/APO-1 antibodies or dexamethasone in ALG-2 depleted cells, but progression of cell death was impaired indicating that ALG-2 is necessary for the apoptotic function of ICE/Ced-3 proteases, however, this Ca^{2+}-binding protein is acting downstream of the proteases (Lacana *et al.* 1997).

By comparing the amino acid sequence of ALG-2 with other members of intracellular Ca^{2+}-binding proteins carrying the EF-hand motif (Kawasaki *et al.* 1998) it was shown that ALG-2 is highly related to the sorcin/grancalcin subfamily of Ca^{2+}-binding proteins containing five EF-hand motifs (Maki *et al.* 1997). The structure of one of the members of this subfamily, the small subunit of calpain, has been determined recently (Blanchard *et al.* 1997; Lin *et al.* 1997). It was described as a dimer in which the two monomers are paired up with each other through the fifth EF-hand which has lost its Ca^{2+}-binding property due to a two residue insertion (Maki *et al.* 1997). Therefore it was proposed that the other members of this subfamily showing the penta-EF-hand motif may also form dimers through the fifth EF-hand in a Ca^{2+}-independent manner (Maki *et al.* 1997) which could provide a new interface for the interaction with possible targets in a similar way as suggested for some of the S100 proteins (Nelson & Chazin 1998). Due to a detailed databank search using the alignment program BLAST (Altschul *et al.* 1990; 1997) we made the interesting observation that ALG-2, originally identified in mice (Vito *et al.* 1996) must have

been highly conserved during evolution since we detected homologues of man, flies and nematodes in the EST database which showed more than 90% (man), 80% (flies) and 70% (nematodes) sequence homology (see Fig. 1). Of special interest was the finding that all 5 EF-hand motifs were conserved in all analysed proteins indicating that ALG-2 must play an important role in Ca^{2+}-dependent functions of apoptosis.

Calcium-dependent endonucleases

One of the consequences during apoptosis in thymocytes is the cleavage of DNA linker regions between nucleosomes (Wyllie 1980) by the activation of Ca^{2+}-dependent endonucleases as demonstrated by McConkey *et al.* (1989). These authors could show that the increase in cytosolic Ca^{2+} preceded DNA fragmentation, that chelation of either extracellular

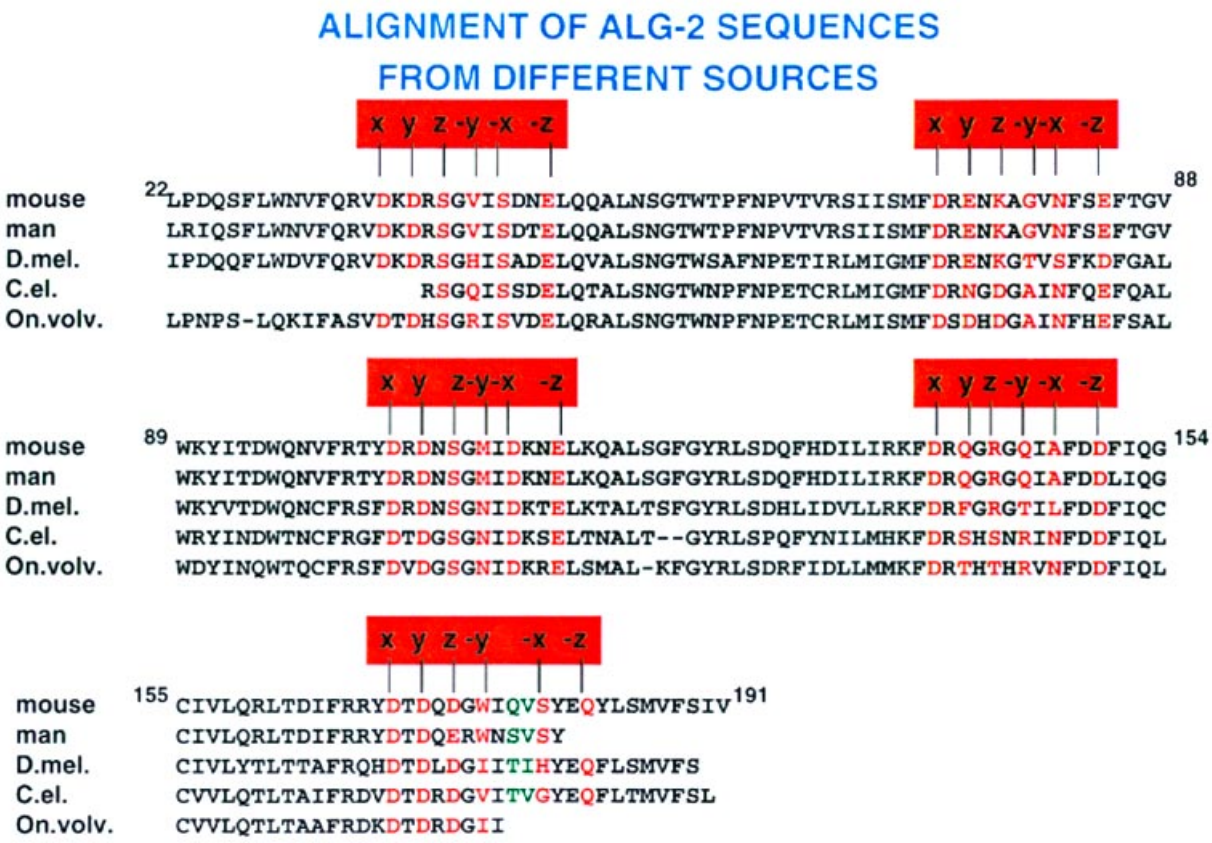

Figure 1. Alignment of the amino acid sequences of the Ca^{2+}-binding proteins ALG-2 from different organisms. The apoptosis-linked gene product ALG-2 as originally determined for the mouse by Vito *et al.* (1996; U49112) was taken as reference to search the different databases by using BLAST as alignment algorithm (Altschul *et al.* 1990; 1997). The mouse protein consists of 191 amino acids as indicated in the Figure, but the alignment was started with amino acid 22, since for most of the other sequences the N-terminal part was not complete. The other sequences came from EST sequences recently deposited in the Databank. The following abbreviations were made: mouse=mus musculus U49112; man = human fetal cDNA AA446365; D.mel.=*drosophila melanogaster* embryonal cDNA AA140803; C.el. = *Caenorhobdatis elegans* genomic DNA of Chromosome I (Y110A7.Contig 88; identical to sequence in M04F3). The C.elegans sequence is translated from the minus strand (frame -1) and starts with nucleotide 6363 and ends with nucleotide 5595. Between nucleotide 6220 and 6171 there is an insert of the following amino acid sequence: V*KNINQNL*K*SFQ. On. volv.=sequence of the cDNA from the nematode *Onchocerca volvulus.;* acc.nr. of the clone is AA934311. The conserved amino acid residues of the EF-Hand loops of the different organisms are indicated in red, the coordination nomenclature was used as introduced by Kretsinger (1975). In the 5th EF-hand domain an insertion was made (enwritten in green) between the positions -y and -x as suggested by Maki *et al.* (1997).

Ca^{2+} by EGTA or intracellular Ca^{2+} by Quin-2 inhibited DNA degradation and that the rise of intracellular Ca^{2+}, and therefore also the induction of apoptosis, was dependent on transcription and/or translation (McConkey *et al.* 1989). In search for the responsible enzyme(s) two interesting observations were made. Peitsch *et al.* (1993) reported endonuclease activity in apoptotic thymocytes producing a characteristic DNA ladder which could be identified as DNAse I. Although normally the enzyme is localized within the endoplasmic reticulum the authors discuss the possibility that the enzyme could be translocated to the nucleus from the perinuclear space due to structural changes of the reticular system associated with apoptotic processes (Peitsch *et al.* 1993).

Another interesting observation was made by Gaido & Cidlowski (1991) who reported on the purification and characterization of a new low-molecular weight Ca^{2+}-dependent endonuclease which they called NUC18. The enzyme is localized in the nucleus, has internucleosomal cleavage properties and is activated by signal transduction pathways known to induce apoptosis. The most remarkable feature of this enzyme, however, is its high sequence similarity to cyclophilins, the molecular targets for the immunosuppressive drug cyclosporin (Montague *et al.* 1994). In addition, the authors also provided evidence that recombinant cyclophilins can carry out Ca^{2+}-dependent nuclease activity indistinguishable from NUC18 suggesting that cyclophilins could play a role in apoptosis (Montague *et al.* 1994; 1997).

Links between Ca^{2+}/Calmodulin-dependent functions and apoptosis

Calmodulin is one of the key mediators of Ca^{2+}-dependent signal transduction pathways in the cell. As described above changes of the intracellular free Ca^{2+} concentrations upon stimuli leading to apoptosis are one of the immediate consequences. Therefore it is not surprising that calmodulin-dependent enzymes may play an important role in apoptotic processes.

In search of genes specifically linked to PCD several Laboratories introduced recently a functional selection strategy of gene cloning (see Deiss & Kimchi 1991; Kimchi 1998). Using this approach the Lab of Kimchi was able to identify several new 'death associated proteins' (DAP) upon treatment of cells with interferon-γ (Cohen *et al.* 1997). A similar approach was used by D'Adamio and his co.workers to isolate the apoptosis linked genes ALG-2 and ALG-3 as described before (Vito *et al.* 1996).

Using this strategy Kimchi and co-workers isolated a number of DAP-proteins (Deiss *et al.* 1995) one of which, DAP-2, is of interest in our context. DAP-2 is a structurally unique 160 kDa calmodulin-dependent serine/threonine protein kinase which contains a number of other domains such as ankyrin repeats, P-loops, cytoskeleton-binding domains and death domains (Cohen *et al.* 1997). The authors could demonstrate that the enzyme phosphorylated myosin light chains in a calmodulin-dependent manner and was associated with the cytoskeleton of the cells. The latter observation could be of special interest since changes in actin microfilament of the cytoskeleton organization preceded the nuclear condensation and segmentation in response to interferon-γ-stimulation or DAP-kinase overexpression (Cohen *et al.* 1997). By ectopic expression in HeLa cells DAP-kinase induced cell death, provided the enzyme was still catalytically active. It was shown that regulation of the kinase activity was essentially dependent on calmodulin since truncating the calmodulin-binding domain resulting in a constitutively active enzyme had a very strong death-inducing effect in overexpression assays (Cohen *et al.* 1997). In contrast, expression of a catalytically inactive mutant in HeLa cells protected these cells from interferon-γ-induced cell death. On the other hand, it was equally important that the catalytically active enzyme was localized correctly on the cytoskeleton. If Cohen *et al.* (1997) overexpressed a truncated, but catalytically active form of the DAP-kinase in HeLa cells, which was mislocalized to the nucleus, this enzyme failed to disrupt the actin microfilament, and, therefore, the cells were not killed.

The enzyme is widely expressed in many cells and seems to play a central role in apoptotic processes since a number of stimuli leading to PCD converge in the activation of this DAP-kinase. A further, very interesting aspect of the role of this enzyme in cellular processes is its possible function as a tumor suppressor gene indicated by the fact that the DAP-kinase could not be detected in a number of lymphomas, leukemia cell lines and cell lines derived from a number of different carcinomas albeit its wide expression in all human and murine tissues tested to date (Kissil *et al.* 1997). In addition, there could be a correlation between the metastatic property of carcinoma cells and DAP-kinase activity, since it was found that high-metastatic lung carcinoma clones lacked DAP-kinase expression whereas their low-metastatic counterparts expressed normal levels of this enzyme (Inbal *et al.* 1997). It was then striking to observe that by restoring normal levels

of DAP-kinase in highly metastatic Lewis carcinoma cells, these cells lost their ability to induce lung metastases after intravenous injection into mice (Inbal *et al.* 1997). Further detailed analysis of the properties of these transfected cells led the authors to the conclusion of a strong correlation between the suppression of metastasis by DAP-kinase and, in turn, the increased sensitivity of those cells towards various death inducing stimuli.

Another calmodulin-dependent enzyme recently described to be involved in the regulation of apoptosis is the phosphatase calcineurin. It is now well established that transcription factors such as NFAT (nuclear factor of activated T-cells) play an important role in the regulation of T-cell proliferation to synthesize IL-2. In order to fulfill its regulatory function NFAT is shuttling between cytosol and nucleus dependent on its phosphorylation state. During T-cell activation NFAT is dephosphorylated by calcineurin and subsequently translocated to the nucleus (see Shibasaki *et al.* 1996 for details). Recently, Shibasaki and McKeon reported that calcineurin could function in calcium-triggered apoptosis in mammalian cells in the absence of growth factors (Shibasaki & McKeon 1995). They further made the interesting observation that co-expression of the protooncogene bcl-2 which normally protects cells against apoptosis efficiently blocks calcineurin-induced cell death. It could also be shown that the calcineurin-induced PCD is calcium independent since a calcium-independent mutant of calcineurin could induce apoptosis in the absence of calcium, but the phosphatase activity was essential for the induction of cell death (Shibasaki & McKeon 1995). In further experiments the group of McKeon could provide convincing evidence that the inhibition of the calcineurin-induced PCD by bcl-2 was due to direct interaction between these two proteins (Shibasaki *et al.* 1997). By forming a tight complex calcineurin is targeted to the localization sites of bcl-2, i.e. to the ER or to the mitochondrial membrane. Important for the interaction between the two proteins was the presence of the so-called BH4 domain at the N-terminus of bcl-2 which is normally used by bcl-2 to form homodimers or heterodimers with related proteins such as Bax (Shibasaki *et al.* 1997). A very important finding was the observation that calcineurin, even if bound to bcl-2, still displayed phosphatase activity, but was unable to promote the nuclear translocation of NFAT, required for induction of IL-2 expression during T-cell activation. Since the BH4 dimerization domain of bcl-2 was also the same domain with which bcl-2 interacts with the kinase raf-1 (Wang *et al.* 1996) the authors propose the possibility that the interaction of a kinase and a phosphatase with the apoptosis protecting protein may reflect a mechanism of bcl-2 regulated cell death.

It thus can be concluded that in programmed cell death calcium plays a pivotal role in mediating a number of key functions. This includes the regulation of a number of enzymes involved in apoptotic processes or, directly, calcium-binding proteins of the EF-hand type such as ALG-2. Mediators of cellular functions often play a decisive role in which direction cells may proceed, whether they differentiate, proliferate or die, and misbalance in these decisions may lead to diseases. Some aspects where calcium could play a crucial role in these decisions have been discussed.

Note added in proof

Recently, R. Sadoul (Geneva, Switzerland) identified in mice a protein which seems to interact specifically with ALG-2 in a calcium-dependent manner (Acc.Nr. AJ005073 in the EMBL data bank). The protein contains 869 amino acid residues, and is highly conserved during evolution since a database search revealed highly homologous transcripts in human (AA337670; 93% homology), D. mel. (AA941497; 64%), *C. elegans* (U73679; 60%), and *S. cerevisiae* (U37364; 45%).

Two features of these predicted proteins are worth noting: they all share a conserved potential tyrosine phosphorylation recognition site – KDNDFI**Y** – for signaling tyrosine kinases, and are proline rich at the C-terminus with several SH3 recognition motifs indicating possible docking sites for SH3 domain-containing proteins. In this context it is also worth mentioning that the homologous protein of budding years, BRO1, has been shown to be involved in vesicle trafficking and docking (Nickas & Yaffe *Mol. Cell Biol. 16,* 2585, 1996; see also Cao et al. *J. Biol. Chem.* **273,** 21077, 1998), a step also important during the recognition process of apoptotic bodies by phagocytotic macrophages.

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