Adenine Nucleotide Translocase-1, a Component of the Permeability Transition Pore, Can Dominantly Induce Apoptosis

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Abstract. Here, we describe the isolation of adenine nucleotide translocase-1 (ANT-1) in a screen for dominant, apoptosis-inducing genes. ANT-1 is a component of the mitochondrial permeability transition complex, a protein aggregate connecting the inner with the outer mitochondrial membrane that has recently been implicated in apoptosis. ANT-1 expression led to all features of apoptosis, such as phenotypic alterations, collapse of the mitochondrial membrane potential, cytochrome c release, caspase activation, and DNA degradation. Both point mutations that impair ANT-1 in its known activity to transport ADP and ATP as well as the NH₂-terminal half of the protein could still induce apoptosis. Interestingly, ANT-2, a highly homologous protein could not lead to cell death, demonstrating the specificity of the signal for apoptosis induction. In contrast to Bax, a proapoptotic Bcl-2 gene, ANT-1 was unable to elicit a form of cell death in yeast. This and the observed repression of apoptosis by the ANT-1–interacting protein cyclophilin D suggest that the suicidal effect of ANT-1 is mediated by specific protein–protein interactions within the permeability transition pore.

Key words: cell death • ATP transport • transfection • apoptosis • membrane potential

Apoptosis is a form of cell death that plays a role in development, tissue homeostasis, and disease (White, 1996). Its induction must be tightly regulated. Otherwise, serious consequences may follow. A hyperactive apoptosis induction might lead to degenerative diseases like Alzheimer’s disease (Loo et al., 1993); a reduced activity can contribute to the multistep process of tumorigenesis since tumor cells are exposed to multiple proapoptotic stimuli (McGill, 1997). Therefore, the induction of apoptosis is governed by an elaborate array of checks and balances in the cell. Eventually, a family of cysteine proteases, the so-called caspases, is activated (Salvesen and Dixit, 1997). These enzymes can cleave specific substrates in the cell that leads to the typical apoptotic phenotype and the self-destruction of the cell.

The first indication that mitochondria play a role in apoptosis induction was the observation that an in vitro system for apoptosis induction required the presence of mitochondria (Newmeyer et al., 1994). The permeability transition pore (PT pore) complex (Zoratti and Szabo, 1995), a protein aggregate that resides in contact sites of the inner and outer mitochondrial membrane, was subsequently identified as being responsible for apoptosis induction (Petit et al., 1995; Zamzami et al., 1995b). Multiple pharmacological stimuli have been shown to activate this complex by as yet unknown means (Petit et al., 1995; Fulda et al., 1998; Zamzami et al., 1998). The activated complex leads to the collapse of the potential over the inner mitochondrial membrane (ΔΨᵢ), swelling of mitochondria, and the generation of oxygen radicals (Zamzami et al., 1995a; Vander Heiden et al., 1997). The subsequent release of apoptogenic caspases (Susin et al., 1999a) and a putative oxidoreductase (Susin et al., 1999b) aids in apoptosis induction.

Many genes involved in apoptosis have the dominant capacity to induce cell death upon overexpression. This feature is even conserved across species (McCarthy and Dixit, 1998). It might be explained by the fact that the overexpressed proteins engage in protein–protein contacts and can thereby create the signal for apoptosis (Yang et al., 1998). Consequently, we have recently developed a screen for dominant, apoptosis-inducing genes (Grimm and Leder, 1997). The screen is based on the iterative transfection of small plasmid pools of a normalized cDNA library protein; H₂A, hemagglutinin; ICAD, inhibitor of caspase-activated DNase; PARP, poly-ADP-ribose polymerase; PT pore, permeability transition pore.
into mammalian cells and the morphological determination of apoptosis induction. Here, we describe the characterization of adenine nucleotide translocase-1 (ANT-1), a central component of the permeability transition pore, which was identified using such a screen.

A NT-1 has recently been shown to be required for apoptosis mediated by Bax, another component of the PT complex (Marzo et al., 1998a). Conversely, a NT-1 can induce apoptosis in a Bax-dependent manner. For this, a NT-1 must be inactivated pharmacologically by the plant glucoside atracyloside, which arrests A NT-1 in a specific conformation and causes PT pore opening (Zoratti et al., 1982; D'avidson and H a lestrap, 1987). However, in our experiments with A NT-1, such a secondary signal is not required for apoptosis induction. A NT-1's apoptosis activity does not seem to depend on its known function for ATP/ATP exchange because several transport inactive mutants could still lead to cell death. Surprisingly, we found that a very homologous protein, the A NT-2 transporter, was inactive for apoptosis induction. Furthermore, a NT-1 could not elicit a form of cell death in yeast. This is in contrast to Bax that directly interacts with A NT-1 and can dominantly induce cell death in yeast. This result suggests that A NT-1-mediated apoptosis induction depends on protein–protein interactions that are specific for mammalian cells. Since A NT-1 is activated by overexpression for apoptosis induction, it is interesting to note that this gene is already highly expressed in mitochondria. This suggests that in a normal cell it must be kept inactive by other proteins of the PT pore, which implies important stoichiometric correlations between the various components of this complex. Consistent with this, we found that cyclophilin D, another component of the PT pore, can repress A NT-1-induced apoptosis. Furthermore, our data could help to explain the observed apoptosis induction in diluted cardiomyopathy (DCM), a degenerative disease of the heart muscle that is marked by a dramatic increase of the expression level of A NT-1 and by excessive apoptosis induction. These findings are, up to now, the most direct genetic evidence that the PT pore can signal apoptosis, and may lead to the molecular elucidation of how this complex can be activated for apoptosis induction.

**Materials and Methods**

**Plasmids**

The hemagglutinin (HA) tag vector (pHA) for the expression of HA fusion proteins was constructed by annealing oligos coding for the HA epitope (YPYDVPDYA) and inserting them between the NotI and XbaI site into the pcDNA 3 vector (Invitrogen Corp.). The correct sequence was verified by sequencing. The point mutants of A NT-1 were engineered by recombinant PCR using suitable primers. A ll amino acid changes were verified by sequencing. Deletion mutagenesis of A NT-1 was achieved with PCR using the same enzyme. For all the PCR reactions, Pwo, a thermolabile polymerase with proofreading function (Roche Diagnostics) was used to amplify the pcDNA3 vector (Invitrogen Corp.). The correct sequence was verified by sequencing. The eukaryotic expression vector for baculovirus p35 has been described (Clem and Miller, 1994). Reverse transcriptase–PCR was used to clone the mouse ANT-2 (5' primer, 5'-ATCGGATCCCATGAACTGTTAGC-3', and 3' primer, 5'-TAGTGGCAATCGCGGC-3') and the human cyclophilin D (5' primer, 5'-ATCGGATCCCATGAACTGTTAGC-3', and 3' primer, 5'-ATCGGATCCCATGAACTGTTAGC-3'). Both were inserted into the pHA vector.

**Cell Cultivation and Transfection**

Human embryonic kidney cells (293T) (Graham et al., 1977; Pear et al., 1993) and BHK cells were kept in DMEM medium supplemented with 5% FCS (Sigma Chemical Co.); all other cell lines were cultivated in DMEM and 10% FCS with the exception of PC3 cells, which were held in RPMI with 10% FCS. All transfections were done by calcium phosphate coprecipitation as described (Roussel et al., 1984), except for PC3 cells which were transfected with Dsper (Roche Diagnostics) and 3T3 cells by using SuperFect (Qiagen). When the green fluorescent protein (GFP) was cotransfected, the indicated amount of pl anter vector (GIBCO BRL) was added to the transfection mix.

**Isolation of Apoptosis-inducing Genes**

m RNA was isolated from 10-wk-old CD1 mice and normalized as described (Grimm and Leder, 1997). The cDNA was inserted into a modified pcDNA 3 vector in which the neomycin resistance gene had been deleted (Grimm and Leder, 1997). The screen for dominant apoptosis inducers was performed essentially as described (Grimm and Leder, 1997). A novel 96-well DNA isolation method allowed a considerably higher throughput. Human 293T cells were used as a read-out cell line since this would allow the isolation of genes whose signals are conserved across species. A apoptosis induction was verified by transfecting the isolated, untagged A NT-1 into mouse L929 and 3T3 cells, the hamster BHK cell line, and human HeLa, MCF-7, and PC3 cells.

**Immunoblotting**

For detecting cytochrome c, 293T cells were transfected with an expression vector for A NT-1. After 18 h, the cells were harvested and the cytoplasts were extracted as described (Ferrari et al., 1998). Protein samples of 30 μg were loaded on SDS–polyacrylamide gels (10%), and then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (A mersham). Western blots were probed with a monoclonal anti cytochrome c antibody (PharMingen) and anti-mouse HRP-conjugated antibody before enhanced chemiluminescence (ECL)–based detection (A mersham). A novel 96-well DNA isolation method allowed a considerably higher throughput. Human 293T cells were used as a read-out cell line since this would allow the isolation of genes whose signals are conserved across species. A apoptosis induction was verified by transfecting the isolated, untagged A NT-1 into mouse L929 and 3T3 cells, the hamster BHK cell line, and human HeLa, MCF-7, and PC3 cells.

**Yeast Methods**

Standard yeast methods were applied (A usubel et al., 1991). For the inducible expression of Bax and A NT-1, the cDNAs were subcloned into pET3p2 (Invitrogen Corp.) in which the B42 fusion moiety was removed.

For induction of the expression constructs, EGY 48 yeast cells (MATα trp1 ura3 his3 trp8 trp1a::lexA operon ulex2) were grown for 8 h in YC medium with 20% galactose and 20% raffinose instead of glucose. The yeast cells were harvested and protoplasts were formed by enzymatic digestion of the cell wall as described (G awaz et al., 1990). T he protein extracts were isolated as described above. Protein samples were separated on 10% polyacrylamide gels and blotted onto PVDF membranes. PARP and cleavage products were detected using a polyclonal anti PARP antibody (R oste Diagnostics), anti-rabbit HRP-conjugated antibody (A mersham) and the ECL system. For the detection of HA-tagged A NT isoforms, mitochondria were isolated by differential centrifugation as described previously (S crieber et al., 1989). A liquots of 50 μg protein were subjected to SDS–PAGE (8% polyacrylamide) and blotted onto PVDF membranes. PARP and cleavage products were detected using a polyclonal anti PARP antibody (R oste Diagnostics), anti-rabbit HRP-conjugated antibody (A mersham) and the ECL system. For the detection of HA-tagged A NT isoforms, mitochondria were isolated by differential centrifugation as described previously (G awaz et al., 1990). 35 S-labeled protein samples were separated on 10% polyacrylamide gels and blotted onto PVDF membranes. Detection was performed using a rat mAb against the influenza virus HA peptide (R oste Diagnostics) and the ECL system (A mersham).
(Research Diagnostics) was used to detect the cytochrome oxidase subunit I.

**Apoptosis Detection**

Low molecular weight DNA from apoptotic cells was isolated and detected as described (Grimm and Leder, 1997). A apoptosis induction was measured by an ELISA (Roche Diagnostics) specific for nucleosomal DNA fragments that are released during apoptosis. The recommendations of the manufacturer were followed. Equal transfection efficiencies were monitored by cotransferring an expression plasmid for GFP. Other apoptosis quantifications were performed by flow cytometry (Bitzer et al., 1999). A cotransfected GFP expression plasmid was used to assess the transfection efficiency. The apoptotic population was put in relation to the percentage of GFP-positive cells. The apoptotic background of the vector control was subtracted to obtain the specific apoptosis induction. Each condition was tested in at least three independent experiments.

**Mitochondrial Membrane Gradient Detection**

HeLa cells were used for the investigation of the membrane potential of mitochondria because they display a better integrity of internal structures after fixation than 293T cells. After transfecting an expression vector for ANT-1 together with a plasmid for GFP, cells were loaded with MitoTracker RedCMXRos (Molecular Probes, Inc.) according to the suggestions of the manufacturer. Images were documented using a Zeiss Axioplan fluorescence microscope. RedCMXRos fluorescence was excited at 546 nm and emission was imaged at >590 nm. GFP fluorescence was excited at 450-490 nm, and emission was monitored at 515-565 nm.

**Results**

**ANT-1 Induces Cell Death**

Using our screen for dominant, apoptosis-inducing genes, we isolated a cDNA whose expression elicited an especially fast and strong apoptosis response in 293T cells. Sequencing revealed that the gene encoded ANT-1, the ADP/ATP translocator protein of the PT pore (Le Quoc and Le Quoc, 1988; Halestrap, 1991). ANT was active in a wide variety of cell types; every cell line tested so far underwent apoptosis after stimulation by ANT-1 (such as L929, 3T3, HeLa, BHK, MCF-7, and PC3). Fig. 1 shows the phenotype of control- and ANT-1–transfected HEK 293T cells. Whereas the vector-transfected cells displayed a normal morphology, ANT-1–expressing cells showed a constricted cytoplasm and blebbing of the plasma membrane.

To assess the temporal relation between the expression of ANT-1 and apoptosis induction, we transfected 293T cells with ANT-1 and investigated apoptosis induction at different time points. Fig. 2 A shows an increase in cell death from 5 to over 50% after 20 h. This correlated with the accumulation of the HA–tagged ANT-1 protein specifically in mitochondrial fractions of the cells (Fig. 2 B). Cytochrome c oxidase was used as a marker protein for the efficient separation of cytoplasmic and mitochondrial protein fractions.

**Figure 1.** ANT-1 expression leads to phenotypic apoptosis induction. The empty vector or an expression plasmid for ANT-1 were transiently transfected into 293T cells. After 16 h, phase-contrast pictures were taken at a 200-fold magnification.

**Figure 2.** Time course of apoptosis induction and protein expression after ANT-1 transfection. (A) Temporal increase of apoptosis induced by ANT-1 expression. 1 μg of the HA–tagged ANT-1 plasmid was transfected into 293T cells for each condition. Quantitative FACS analysis of sub-G1–positive cells was performed at the indicated time points. The specific apoptosis induction above background is shown as a percentage of apoptotic cells relative to all transfected cells. The means and the SDs are given for each experiment. (B) Transfected ANT-1 progressively accumulates in mitochondria. Cytoplasmic and mitochondrial cell fractions were prepared from aliquots taken from the samples under A. 35 μg of each were subjected to SDS-PAGE and subsequent immunoblot analysis using an anti–HA tag antibody to specifically detect the transfected ANT-1. The membrane was stripped and reprobed with an mAb against subunit I of cytochrome c oxidase (COX I) to demonstrate the purity of the mitochondrial preparations.
Since ANT-1 is a component of the PT pore whose activation leads to a dissipation of the proton gradient $\Delta \Psi_m$ over the inner mitochondrial membrane (Le Quoc and Le Quoc, 1988; Halestrap, 1991), we tested ANT-1–transfected cells for this membrane gradient. To this end, we cotransfected ANT-1 and an expression vector for GFP into HeLa cells. The membrane gradient in transfected and, therefore, GFP-positive cells was assayed with CMXRos, a dye whose fluorescence is dependent on an intact membrane potential. Fig. 3 A shows that ANT-1–expressing cells displayed a reduced mitochondrial fluorescence with this dye. This differential staining is a very early feature of ANT-1–mediated apoptosis induction since it could be observed in cells that did not yet display an altered phenotype.

One of the consequences of mitochondrial damage is the release of cytochrome c from the inner mitochondrial membrane space (Kluck et al., 1997; Yang et al., 1997). Therefore, we assayed the cytoplasm of ANT-1–transfected cells for the presence of cytochrome c. Using a Western blot, we could detect this protein in the cytoplasmic fraction of ANT-1–transfected cells (Fig. 3 B). The released cytochrome c can bind Apaf-1, a mammalian homologue of the Caenorhabditis elegans gene ced-4 that leads to the aggregation and activation of downstream caspases (Srinivasula et al., 1998). Its degradation leads to the activation of CAD and the internucleosomal degradation of the DNA, a well-known biochemical sign of apoptosis. To investigate the status of the DNA, we transfected 293T cells with a control vector or ANT-1 and isolated the low molecular weight DNA. Only ANT-1–transfected cells displayed the typical DNA ladder (Fig. 4 A). We also investigated the status of PARP, one of the best known caspase substrates, in ANT-1–transfected cells. A Western blot for PARP (Fig. 4 B) revealed that ANT-1–transfected cells degraded PARP and generated the expected protein fragments. Since we have also observed apoptosis induction in MCF-7 cells (data not shown), a cell line that does not express caspase-3 (Janicke et al., 1998), we conclude that ANT-1–induced apoptosis is not dependent on this particular isoenzyme. According to a recent report (Marzo et al., 1998a), Bax, a member of the Bcl-2 gene family, interacts directly with ANT-1 and is also known to induce apoptosis (Oltvai et al., 1993). However, caspase activation does not seem to be a necessary event for Bax-induced cell death (Xiang et al., 1996). Therefore, we wanted to know whether caspase activation is required for ANT-1 apoptosis induction. To address this, we cotransfected ANT-1 and an expression vector for p35, a broad-range caspase inhibitor from baculovirus (Clem and Miller, 1994). A quantitative apoptosis assay revealed a sixfold reduction in apoptosis induction when p35 was cotransfected (Fig. 4 B).
These results show that ANT-1 leads to all phenotypic and biochemical alterations associated with apoptosis.

**The NH2-terminal Half of ANT-1 Is Sufficient for Apoptosis Induction**

ANT-1 was originally described as an ADP/ATP exchange factor (Riccio et al., 1975). To assess whether the transport activity of ANT-1 is required for its apoptosis induction, we generated six different point mutations, all of which have been shown to impede ANT-1's activity to transport ADP and ATP (Muller et al., 1996; Muller et al., 1997). However, upon transfection into 293T cells, we have not observed any difference relative to the wild type (WT) in their potential to induce apoptosis as measured by the release of internucleosomal DNA fragments (Fig. 5 A).

ANT-1 is a member of the mitochondrial carrier family that consists of proteins with three 100-residue repeats comprising alternating regions of hydrophilic and hydrophobic residues. An integral membrane protein, they span the membrane six times, interrupted by short amphipathic helices that are likely to form the transport active moiety.
ANT-2 Is Unable to Induce Apoptosis

The PT pore complex is supposed to function by the conversion of the specific ADP/ATP exchange transporter into an unselective pore (Zoratti and Szabo, 1995). However, since over half of the ANT-1 protein is dispensable for apoptosis induction, we wanted to know whether ANT-1 induces apoptosis by forming an unselective pore on its own. Therefore, we assessed the specificity of apoptosis induction by ANT-1. To this end, we made use of ANT-2, an over 90% identical gene to ANT-1 which is likewise ubiquitously expressed (Doerner et al., 1997a). Transfection of ANT-2 did not lead to apoptosis induction as measured by fluorescence-activated cell sorter (FACS) analysis (Fig. 6 A). The correct mitochondrial localization and efficient expression of ANT-2 was verified by Western blot (Fig. 6 B).

Growth of Yeast Cells Is Not Affected by ANT-1

Bax, a member of the Bcl-2 gene family, can induce a form of cell death by overexpression in yeast cells (Greenhalf et al., 1996; Zha et al., 1996; Jurgensmeier et al., 1997), probably by activating ANT-1 and the PT pore (Greenhalf et al., 1996; Marzo et al., 1998a). Therefore, we wanted to determine whether ANT-1 has the same capacity. To address this, we subcloned ANT-1 and Bax into a galactose- and raffinose-inducible yeast vector. The induction of these genes on suitable agar plates revealed that Bax led to a reduction in the growth of these cells, indicating cell death. In contrast, ANT-1–expressing yeast cells were completely unaffected and grew as well as on the control plate (Fig. 7 A). A Western blot revealed the efficient expression of Bax and ANT-1 in yeast cells. (Fig. 7 B)

Cyclophilin D Can Repress Apoptosis Induced by ANT-1

Our experiments with ANT-2 and cell death in yeast suggested that ANT-1’s apoptotic activity is mediated by specific protein–protein interactions rather than by forming an active PT pore on its own. Nevertheless, to check whether ANT-1 expression activates the endogenous PT pore, we made use of bongkrekic acid, a specific inhibitor
Discussion

In this study, we have described the dominant activity of A NT-1, a central component of the PT pore in mitochondria, to induce apoptosis upon overexpression. This is the first report that shows such an activity of a component of the PT pore in the inner mitochondrial membrane. These findings have important consequences for the normal state of the cell: A NT-1 is already one of the most abundant proteins in mitochondria and makes up ~10% of all proteins in the inner mitochondrial membrane (K lingenberg, 1993). This means that its dominant activity for apoptosis induction must be repressed by other proteins in a non-apoptotic cell (see below).

In addition, A NT-1 is specifically and dramatically up-regulated in heart tissue of patients with DCM (Schultheiss et al., 1996; Doerner et al., 1997b). DCM is a myocardial disease of unknown etiology that is characterized by a reduced myocardial contractility and ventricular dilatation. Its estimated prevalence is 36 in 100,000 and, therefore, represents a major cause of congestive heart failure (Dec and Fuster, 1994). Interestingly enough, excessive apoptosis can be observed in tissues from DCM patients (Narula et al., 1996; Y ao et al., 1996; Y amamura et al., 1999). Even though different tissues with distinct A NT isoform levels might be differentially sensitive to A NT-1 expression, we would like to propose that this pathological apoptosis induction is mediated by the increase in A NT-1. Therefore, our studies might lead to an explanation and possibly to a treatment of this disease.

Our initial hypothesis for the apoptosis induction by A NT-1 was that it is was caused by its known function of shutting A TP and A DP over the mitochondrial membrane. However, the apoptosis potential of A NT-1 seems not to be dependent on its activity to transport A DP and A TP, because a panel of point mutants that are inactive for this transport could still induce apoptosis (Fig. 5). While the transfect A NT-1 can, therefore, be impaired for nucleotide exchange, the A DP/A TP transport activity of the endogenous A NT-1 might still be relevant for apoptosis induction and be inhibited by the overexpressed A NT-1.

The activation of the PT pore for apoptosis induction is thought to be mediated by the PT pore becoming an unspecific pore and releasing apoptogenic proteins and oxygen radicals (Marzo et al., 1998b). Isolated A NT-1 has been shown to form an unspecific, Ca2+-dependent pore (Brustovetsky and K lingenberg, 1996; Ruck et al., 1998). Furthermore, A NT-1 can be activated for apoptosis induction by atractylloside, a plant glucoside that binds to A NT-1, stabilizes a specific conformation, and favors pore opening (Z oratti et al., 1982; Davidson and Halestrap, 1987). Therefore, our second hypothesis was that overexpressed A NT-1 forms such an unspecific pore and thereby reproduces an activated PT pore. However, several lines of evidence suggest that overexpressed A NT-1 does not form an unspecific pore. First, deletion studies with A NT-1 showed that the C OOH-terminal half of the molecule, including two transmembrane domains, is dispensable for apoptosis induction (Fig. 5 B). It is hard to imagine how such a truncated molecule could still form a membrane channel. Second, A NT-2 was inactive for apoptosis induction (Fig. 6). Since this protein is over 90% identical to A NT-1, it should also be capable of assembling into an unspecific pore. Thirdly, if A NT-1 overexpression was sufficient to form an unspecific channel, one would expect that A NT-1 could induce cell death also in yeast. However, our results show that A NT-1 was inactive in yeast (Fig. 7).

Therefore, we would like to propose a model (Fig. 9) in which A NT-1, rather than forming an activated PT pore by itself, stimulates the endogenous PT pore for apoptosis induction. This is achieved by titrating out inhibitors of this complex that activate the PT pore and induce apoptosis. One of these inhibitors could be cyclophilin D, which we have shown to be able to repress A NT-1-induced apoptosis (Fig. 8 B). Alternatively, cyclophilin D might also mask an interaction domain in A NT-1 for another protein that is a repressor for the PT pore. Therefore, this protein is not titrated out any more and the PT pore is not activated.

This interpretation of our results suggests that the stoichiometry of the proteins in the PT pore complex is an important factor for its inactive state. The inner mitochondrial membrane would, therefore, mirror the condition in
the outer membrane where the relative abundance of pro- and anti-apoptotic proteins (e.g., the Bcl-2 family members) determines apoptosis induction (Korsmeyer, 1995). According to this model, ANT-1, instead of forming an unspecific pore, would mediate apoptosis by specific protein–protein interactions. Different affinities for these proteins could, therefore, account for ANT-2's inactivity to induce apoptosis.

Other proteins in our model would facilitate apoptosis through ANT-1. Their existence is postulated because isolated ANT proteins require the presence of Ca²⁺ and, therefore, cannot function as an unspecific pore on their own (Brustovetsky and Klingerberg, 1996; Ruck et al., 1998). These activators might not be titrated out by ANT-1 because their affinity to ANT-1 is dependent on the interaction with other proteins like, for example, Bax. Our data from yeast might be explained by assuming that the interaction of ANT-1 with the inhibitors is not conserved between yeast and mammals, and that, therefore, these inhibitors are not titrated out. Alternatively, or in addition, the inducer proteins in the mammalian complex are not present in yeast or their interaction is regulated differently.

Based on previous studies with Bax (Oltvai et al., 1993) and on this report about ANT-1, both proteins can induce apoptosis upon overexpression. It was further shown that they interact directly, and that the activation of ANT-1 for apoptosis by the plant glucoside atractyloside required the presence of Bax and vice versa (Marzo et al., 1998a). However, there are interesting differences in their capacity for apoptosis induction: Bax can induce a form of cell death in yeast, whereas ANT-1 is inactive (Fig. 7). A so no Bcl-2-like gene and, therefore, no Bax homologue is found in the yeast genome. Bax might represent ANT-1's missing interaction partner in yeast. The association between Bax and ANT has been shown to be mediated by residues 105–156 of ANT (Marzo et al., 1998a). These sequences are also required for ANT-1 apoptosis induction (Fig. 5). This direct Bax–ANT interaction might suggest that Bax is activated by ANT-1 expression. However, Bax-induced apoptosis does not seem to rely on caspase activation, even though this protease family is activated when Bax is overexpressed (Xiang et al., 1996). ANT-1, on the other hand, requires caspase activation as shown through the inhibition by baculovirus p35 (Fig. 4 C). Furthermore, and most importantly, even though ANT-2 has been shown to interact with Bax (Marzo et al., 1998a), it is unable to induce apoptosis (Fig. 6).

In addition, Bax might not be available for interaction with the transfected ANT-1 in the cell because the endogenous ANT-1 is already a very abundant protein and might complex all endogenous Bax. For these reasons, we believe that the transfected ANT-1 does not activate the endogenous Bax, and that overexpressed ANT-1 induces apoptosis in a different way than atractyloside-activated ANT. Taken together, ANT-1's apoptosis induction as described in this report shows how a protein that performs the essential biological function to facilitate the presence of a TP in the cytoplasm can also, under certain conditions such as in DCM, turn into a protein that elicits a deadly signal for the cell.

We would like to thank U. Cramer and F. Neudecker for excellent technical assistance, Dr. M. Klingerberg for the anti-A NT-1 serum, Dr. J.A. Dúne for providing bongkrekic acid, A. K hilhuber for suggestions concerning the manuscript, and the Bavarian Government and Roche Diagnostics for their support.

Submitted: 14 June 1999
Revised: 3 November 1999
Accepted: 9 November 1999

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Figure 9. Hypothetical model for ANT-1's apoptosis-inducing activity. The PT pore complex is depicted with its different components in the outer and inner mitochondrial membrane. According to the model, ANT-1 can interact with proteins that repress the PT pore (indicated as hatched circles) and with proteins that facilitate apoptosis (indicated in dark shading). Overexpressed ANT-1 proteins (shown on the right and left) do not form an activated PT pore on their own, but rather titrate out the repressors of the endogenous PT pore that activate the PT pore. For this activation, additional proteins are needed that are not titrated out by hyperexpressed ANT-1 because they need additional interaction partners for their affinity to ANT-1. See text for a more detailed discussion of this model.
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