Cyclophilin D, a Component of the Permeability Transition-Pore, Is an Apoptosis Repressor

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ABSTRACT

The permeability transition (PT)-pore is an important proapoptotic protein complex in mitochondria. Although it is activated by many signals for apoptosis induction, the role of its various subunits in cell death induction has remained largely unknown. We found that of its components, only the voltage-dependent anion channel in the outer mitochondrial membrane and the adenine nucleotide translocator-1 (ANT-1), a PT-pore subunit of the inner membrane, are apoptosis inducers. We also report that ANT-1’s direct interactor, cyclophilin D, can specifically repress ANT-1-induced apoptosis. In addition, cotransfection experiments revealed that for a diverse range of apoptosis inducers, cyclophilin D shows the same repression profile as the compound bongkrekic acid, a specific inhibitor of the PT-pore. This activity seems to be independent of its chaperone activity, the only known function of cyclophilin D to date. Importantly, cyclophilin D is specifically up-regulated in human tumors of the breast, ovary, and uterus, suggesting that inhibition of the PT-pore via up-regulation of cyclophilin D plays a role in tumorigenesis.

INTRODUCTION

Apoptosis, or controlled cell death, is a complex process whose regulation requires the cell to integrate a variety of different signals, both pro- and antiapoptotic. In particular, mitochondria have recently been described to be integrators of such signaling pathways (1). The permeability transition (PT)-pore is a mitochondrial protein complex on which many signals for cell death converge (2, 3). Because it is regulating cell death by activating downstream effectors, this protein aggregate can be regarded as a sensor for apoptosis. The PT-pore connects the inner mitochondrial membrane with the outer mitochondrial membrane. Whereas a molecular structure of this complex is still missing, biochemical data suggest that it is composed of a multitude of different components (4). The known constituents of the PT-pore are the voltage-dependent anion channel (VDAC) and the peripheral benzodiazepine receptor in the outer mitochondrial membrane. Two reports have also implicated the proapoptotic Bcl-2-protein Bax to be part of this complex in the outer membrane (5, 6). Furthermore, creatine kinase is described to associate with PT-pore subunits in the intermembrane space. The inner mitochondrial membrane harbors the PT-pore component adenine nucleotide translocator-1 (ANT-1), which is a transport protein for ADP and ATP. Cyclophilin D, a member of the cyclophilin family of chaperones, binds directly to ANT-1. In addition to these known subunits, the PT-pore could comprise further components, possibly explaining why a diverse range of cell death stimuli can activate this protein complex (4). However, there seem to exist some proteins, most prominently among them the proapoptotic Bax protein (7), that appear to be able to cause cell death without recruitment of the PT-pore.

The point of no return for cell death induction by mitochondria seems to be the disintegration of the outer membrane of these organelles and the release of proapoptotic factors such as apoptosis-inducing factor (8), Smac/Diablo (9, 10), or cytochrome c (11). This can be achieved by converting the PT-pore into a nonspecific pore that leads to osmotic swelling of the inner mitochondrial membrane and to the rupture of the outer membrane, providing the gate for the release of proapoptotic proteins from mitochondria (1). However, a molecular description of how the PT-pore is activated for apoptosis is still lacking. In particular, the respective functions of the components of this protein complex for apoptosis induction or repression have not been determined.

The repression of apoptosis is a hallmark of tumorigenesis (12). In line with this, the PT-pore has been implicated in the regulation of apoptosis in tumors. Most of these reports describe the PT-pore as a target for anticancer drugs (13). Moreover, some associated proteins of the PT-pore such as Bax and Bcl-2 have been implicated as tumor suppressor proteins and oncogenes, respectively (14, 15).

The dominant activity of genes to induce or inhibit apoptosis on ectopic expression very often correlates with the function of their endogenous proteins to mediate or repress a cell death signal. It is possible that this is due to the fact that mostly protein-protein interactions are responsible for mediating the apoptosis signal within the cell. Based on this assumption, we have established a screen for apoptosis-inducing genes that are activated on overexpression (16). In a previous report, we have described the apoptosis-inducing activity of ANT-1 (17) that was isolated using this screen. In line with our original hypothesis, ANT-1 has been shown to mediate apoptosis induction via the PT-pore (18). In addition, this gene is dramatically up-regulated in dilated cardiomyopathy, a prevalent degenerative heart disease (19) that is characterized by an excess of apoptosis in myocytes (20). Further investigation of ANT-1-induced apoptosis could therefore become relevant for the treatment of this fatal disease. In the present study, we have continued the molecular analysis of ANT-1-induced cell death in the context of the PT-pore and found that cyclophilin D is a repressor of apoptosis that is up-regulated in hormone-dependent human cancers.

MATERIALS AND METHODS

Materials. All fine chemicals were from Sigma, unless otherwise specified. The pan-caspase inhibitor N-benzylcarboxy-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was obtained from Enzyme Systems.

Cell Culture. Human embryonic kidney cells (293T) and HeLa cells were kept in DMEM (Sigma) supplemented with 5% and 10% FCS, respectively. For the generation of HeLa cells deficient in mitochondrial DNA (HeLa ρ0 cells), cells were incubated for 2 months in 100 μg/ml ethidium bromide in medium supplemented with 50 μg/ml uridine and 100 μg/ml pyruvate (21). 293T, HeLa, and HeLa ρ0 cells were transfected with the calcium phosphate coprecipitation method as described previously (17).

Constructs. The cDNAs of the peripheral benzodiazepine receptor, caspase-8, ANT-2, cyclophilin D, VDAC-I, VDAC-II, creatine kinase, and the mitochondrial chaperones ρ10 and ρ60 were isolated by PCR and subcloned in the mammalian expression vector pcDNA3 (Invitrogen). They were all control sequenced. The expression construct for RIP has been described previously (22). The caspase-2 vector was a gift from S. Kumar. The double point mutant of cyclophilin D (CyboD*R97A/H168Q) was generated by PCR using the Pwo polymerase, which has proofreading activity and was control sequenced.

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Apoptosis Detection. Apoptosis was quantified by determining the amount of sub-G1 DNA with flow cytometry [FACSCalibur; Becton Dickinson (23)]. Briefly, cells were trypsinized, washed, and resuspended in PBS. After the addition of 1.5 volumes of hypotonic propidium iodide buffer [20 μg/ml propidium iodide, 0.1% (w/v) sodium citrate, and 0.1% Triton in PBS], fluorescence-activated cell-sorting analysis was performed. A cotransfected green fluorescent protein (GFP) expression plasmid (pLantern; Life Technologies, Inc.) was used to assess the transfection efficiency. The apoptotic population was measured in relation to the percentage of green fluorescent protein-positive cells. Each condition was tested in at least three independent experiments.

Immunoblotting. For detecting protein expression, cells were harvested by trypsinization, washed with PBS, and lysed in Triton X buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] for 10 min on ice. After centrifugation at 14,000 × g in a microfuge, supernatants were obtained as cytoplasmic extracts that were quantified for protein content using the Bradford reagent (Bio-Rad). Equal amounts of protein were loaded on a 15% SDS-polyacrylamide gel after boiling with 2× sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol, and 0.05% bromphenol blue], separated in an electric field, and transferred to a polyvinylidene difluoride membrane in a semidry blotting device. The membrane was blocked for 1 h with 5% dry milk powder in Tris-buffered saline-

Northern Hybridization. For hybridization of the Matched Tumor/Normal Profiling Array (Clontech), the coding sequence of cyclophilin D was excised from the expression plasmid and labeled with 5′-[α-32P]dCTP using the RediPrime prime labeling kit (Amersham) with 200 ng of plasmid and 50 μCi of [32P]dCTP. The blots were prehybridized for 3 h at 65°C in 40 ml of hybridization buffer (6× SSC, 5× Denhardt’s reagent, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA), 10 ml of a 50% dextran sulfate solution were added, and, finally, the labeled probe (approximately 3 × 10⁷ cpm) was added to the hybridization solution and incubated at 62°C for 6 h. The blot was washed with buffer 1 (2× SSC and 0.5% SDS), buffer 2 (1× SSC and 0.5% SDS), and buffer 3 (0.5× SSC and 0.5% SDS). The blot was exposed for a BAS 2500 phosphorimager screen (Fuji-Films, Heidelberg, Germany). The signals were analyzed and quantified using Image Gauge V3.01 software.

ANT-1 Transport Activity Measurement. The ADP/ATP transport activity of ANT-1 was measured 24 h after transfecting ANT-1 (or β-galactosidase as a control) by a modified protocol (24). Briefly, the cells were permeabized with 12.5 μM digitonin for 4 min, washed, and resuspended in a buffer containing 125 μM ADP to stimulate the antiport of ATP. The ATP that was released into the supernatant was then determined by an enzymatic reaction (25).

Detection of Mitochondrial Membrane Potential (ΔΨ). The mitochondrial membrane potential was assayed by 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes Inc.) Briefly, the transiently transfected cells were harvested and incubated with 0.5 nm 3,3′-dihexyloxacarbocyanine iodide and 2.5 μM ionomycin and analyzed by flow cytometry (FACSCalibur; Becton Dickin-son).

RESULTS

Characterization of ANT-1-Induced Apoptosis. To assess the contribution of the various PT-pore subunits for apoptosis induction, we isolated the known components of the PT-pore by reverse transcription-PCR and transfected them into 293T cells. A quantitative fluorescence-activated cell-sorting analysis revealed that ANT-1 could potently induce apoptosis (Fig. 1A). VDAC-I and VDAC-II, two components of the outer mitochondrial membrane, could also cause cell death but did so less efficiently. In contrast, the other subunits of this proapoptopic protein complex remained inactive for cell death induc-tion. A Western blot showed the efficient expression of the various PT-pore components (data not shown). The difference between ANT-1, a powerful cell death inducer, and ANT-2, a gene completely inactive for cell death induction, was especially striking, given the 90% identity of these two proteins. It is therefore unlikely that nonspecific reactions such as the misfolding of ANT-1 proteins could be responsible for apoptosis induction. Furthermore, after ANT-1 transfection, we observed a 28% increase in the transport of ATP after antipot with ADP (data not shown) over control-transfected cells, suggesting that the ANT-1 protein is correctly folded in the membrane. The different behavior of ANT-1 and ANT-2 facilitated delineation of the domain(s) necessary for cell death induction in ANT-1. To this end, we generated a number of swap mutants that replaced progressively longer sequences beginning at the NH2 terminus of ANT-1 by the corresponding domains of ANT-2. After transfection, apoptosis was quantified and compared. Fig. 1B reveals that four swap mutants (Ex 17, Ex 107, Ex 170, and Ex 207) led to a drop in apoptosis induction when compared with their preceding mutants. According to its proposed structure (26) ANT-1 traverses the membrane six times and thereby forms five intermittent loops. Accordingly, the NH2 terminus and the COOH terminus are proposed to protrude into the intermembrane space of mitochondria. The mapping experiment therefore characterizes four domains in ANT-1 that contribute to apoptosis induction: the very NH2 terminus; and the second, third, and fourth loop. Of note, the exchange of the third, matrix-restricted loop led to the biggest decline in cell death induction. 

The respiratory chain is supposed to be responsible for the generation of reactive oxygen species that are frequently formed during apoptosis and promote cell death inactivation (27). Unrestricted reactive oxygen intermediates formation in mitochondria can cause dilated cardiomyopathy (28), a disease in which ANT-1 is strongly up-regulated (19). We therefore wanted to determine the contribution of the respiratory chain to ANT-1-induced apoptosis. We created HeLa ρ0 cells that are deficient in the respiratory chain by incubating the cells with low concentrations of ethidium bromide for a prolonged period of time. Fig. 1C shows that these cells are dependent on uridine addition, which is indicative of cells with an inactive respiratory chain (21). In addition, we observed that cytochrome oxidase that is encoded by the mitochondrial DNA is absent in these cells. In the transfections we used conditions that resulted in a relatively weak extent of cell death to be able to assess even slight differences in apoptosis induction. The bottom panel of Fig. 1C reveals that ANT-1 could induce apoptosis as potently in HeLa ρ0 cells as in wild-type HeLa cells. RIP, a death domain-containing protein kinase (22), was used as a control apoptosis inducer and was likewise equally efficient for cell death induction in these two cell lines. However, tumor necrosis factor (TNF) induced a much weaker apoptosis in HeLa Cells (21). In addition, we observed that cytochrome oxidase that is encoded by the mitochondrial DNA is absent in these cells. In the transfections we used conditions that resulted in a relatively weak extent of cell death to be able to assess even slight differences in apoptosis induction. The bottom panel of Fig. 1C reveals that ANT-1 could induce apoptosis as potently in HeLa ρ0 cells as in wild-type HeLa cells. RIP, a death domain-containing protein kinase (22), was used as a control apoptosis inducer and was likewise equally efficient for cell death induction in these two cell lines. However, tumor necrosis factor (TNF) induced a much weaker apoptosis in HeLa ρ0 cells. In addition, cytochrome bl, a component of complex II of the respiratory chain that was isolated as an apoptosis inducer, was inactive in these cells (29).

Cyclophilin D Specifically Reduces ANT-1-Induced Apoptosis. The above result, together with the mapping experiment, suggested that specific protein-protein interactions, not the transport activity of ANT-1, are responsible for apoptosis induction. A large body of work on apoptosis suggests that it is mediated by specific protein-protein interactions. Consequently, both repressors and inducers exhibit their function on overexpression. We were therefore interested whether other components of the PT-pore that we have shown to be inactive for apoptosis induction (Fig. 1A) could influence ANT-1-induced apoptosis when coexpressed with ANT-1. Fig. 2A reveals that only creatine kinase and cyclophilin D could significantly reduce cell death. Because cyclophilin D was the strongest repressor, we focused...
Cyclophilin D is a Repressor of Apoptosis

Fig. 1. Characterization of adenine nucleotide translocator-1 (ANT-1)-induced apoptosis. A, test of the various components of the permeability transition-pore for apoptosis induction. Five μg of the respective expression plasmids (adenine nucleotide translocator-2, ANT-2; voltage-dependent anion channel-1, VDAC 1; creatine kinase, CK; peripheral benzodiazepine receptor, PBR; cyclophilin D, CycD) together with 1 μg of green fluorescent protein expression plasmid were transfected into 293T cells. For its high efficiency in apoptosis induction, only ANT-1 was used with 1 and 4 μg of a luciferase construct. Apoptosis was quantified by staining with propidium iodide and fluorescence-activated cell-sorting analysis 18 h after transfection. The data were normalized to the transfection efficiency as assessed by green fluorescent protein fluorescence. The means and SDs of three independent experiments are shown. B, mapping of the apoptosis-inducing domains in ANT-1 by swap mutants of ANT-1 and ANT-2. One μg of different swap mutants that exchange progressively longer sequences beginning at the NH2 terminus of ANT-1 with the corresponding sequences of ANT-2 were generated and transfected into 293T cells in 6-well plates. A schematic drawing shows the ANT-2 sequences in light shading and the ANT-1 domains in dark shading. The position of the last residue of ANT-2 sequence in the exchange (Ex) mutants are given. One μg of an expression plasmid that allowed us to normalize the apoptosis rate to the transfection efficiency was cotransfected. Apoptosis was detected 18 h after transfection by propidium iodide staining and quantified by fluorescence-activated cell-sorting analysis. The means and SDs of three independent transfections for each construct are shown. C, independence of the ANT-1 apoptosis induction from an intact respiratory chain. HeLa ρ0 cells that are deficient in the respiratory chain were generated by prolonged incubation in ethidium bromide. The cells were then investigated for their dependence on addition of uridine to the medium, which is a marker for the absence of mitochondrial DNA (top panels). Scale bar, 100 μm. The presence of the mitochondrially encoded cytochrome oxidase subunit 1 (COX I) was investigated in a RNA blot of HeLa ρ0 cells, and a control against β-actin showed equal loading of the lanes (middle panels). Comparison of ANT-1- and RIP-induced apoptosis in wild-type HeLa and HeLa ρ0 cells is shown (bottom panels). Cells were transfected with the indicated expression plasmids (0.2 μg of ANT-1 or RIP plasmid with 50 ng of green fluorescent protein expression plasmid) and apoptosis was quantified by a fluorescence-activated cell-sorting analysis after 30 h.

on this gene for further analysis. We also observed a repression of ANT-1-induced apoptosis in HeLa cells by cyclophilin D (data not shown). Because both proteins, cyclophilin D and ANT-1, are located in mitochondria, we wanted to address the question of whether cotransfected cyclophilin D reduces the import of ANT-1 and therefore leads to a reduction of apoptosis. Fig. 2B shows that even a slightly increased amount of ANT-1 protein could be found in mitochondrial extracts when cyclophilin D was cotransfected.

Based on the sequence homology, cyclophilin D is a member of the cyclophilin family of chaperones, some of which have been found to regulate apoptosis, such as Hsp70 (30) and Hsp90 (31). In fact, cyclophilin D has been shown to possess peptidylprolyl isomerase activity (32). Hence, we were interested in determining whether the peptidylprolyl isomerase activity of cyclophilin D is important for apoptosis reduction. A construct with two point mutations near and in the hydrophobic binding pocket that have both been shown to abrogate the peptidylprolyl isomerase activity in cyclophilins (33) was generated. As Fig. 2C demonstrates, this construct could still reduce ANT-1-induced apoptosis. In addition, we observed that the cotransfection of two other mitochondrial chaperones, p10 and p60, which together form an active protein folding complex in mitochondria (34), were inactive for apoptosis repression (Fig. 2D). These results indicate that the two activities of cyclophilin D, apoptosis regulation and protein folding, can be functionally separated.

Common to pathways that stimulate the PT-pore for apoptosis induction is the breakdown of membrane potential (ΔΨ) over the inner mitochondrial membrane (35). We therefore tested this membrane potential using 3,3′-dihexyloxacarbocyanine iodide, which accumulates in mitochondria with an intact membrane gradient. The Ca2+-specific ionophore ionomycin served as a PT-pore inducer. It is known that Ca2+ influx activates the PT-pore (3). Fig. 2E shows that on cyclophilin D expression, the number of cells with a reduced mitochondrial membrane potential was decreased.

Apoptosis Repression by Cyclophilin D Correlates with Cell Death Reduction by Bongkrekic Acid (BA), a Specific PT-Pore Inhibitor. To assess the specificity of cyclophilin D for the repression of ANT-1-induced cell death, we have cotransfected this gene...
with a number of proapoptotic genes and compared apoptosis repression by cyclophilin D with cell death inhibition by BA. BA is a compound that specifically associates with ANT-1 and inhibits the activation of the PT-pore (36). As a further control, we used Bcl-2 and Bcl-XL, two known inhibitors of the Bcl-2 gene family. First, we tested two proapoptotic components of the PT-pore for their repression profile. Fig. 3, A and B, shows that whereas ANT-1-induced apoptosis could efficiently be repressed by cyclophilin D, cell death by Bax expression was only moderately inhibited by cotransfected cyclophilin D. Likewise, BA could potently diminish apoptosis induced by ANT-1 but was only a weak inhibitor for Bax (Fig. 3C). Of note, Bcl-XL behaved differently with these two apoptosis inducers: it was a strong repressor of Bax-induced apoptosis but was inactive for ANT-1-mediated cell death. Subsequently, we tested apoptosis inducers that are not components of the PT-pore and might therefore activate this protein complex in a more indirect way. Caspase-2 is a caspase that can be found in the nucleus and the Golgi complex (37) and is activated by a diverse range of signals (38). Recently, it was shown that this protease mediates cytotoxic stress directly rather than only amplifying a mitochondrial cell death signal (39). Overexpressed caspase-2 was not inhibitable by both cotransfected cyclophilin D and BA (Fig. 4A). Caspase-8 can be found in the active TNF- and the
Fas-receptor complex (40) but in addition seems to initiate a proapoptotic signal at the endoplasmic reticulum (41). Cell death by this protease could be repressed by about 50% by both cotransfected cyclophilin D and BA treatment (Fig. 4B). RIP, another component of the TNF receptor complex, could only be partially inhibited by cyclophilin D. This is mirrored by a moderate reduction of RIP-mediated cell death after application of BA (Fig. 4C). Consequently, BA and cyclophilin D correlate in their activity to diminish apoptosis induced by both components of the PT-pore and apoptosis inducers residing at other locations in the cell.

Cyclophilin D Is Up-Regulated in Specific Human Tumors. Because apoptosis is suppressed in almost every tumor to overcome the many adverse conditions tumor cells encounter, cell death repression is supposed to be a hallmark of cancer cells (12). Consequently, cyclophilin D was also active as an apoptosis repressor in HeLa cells. Both apoptosis inducers, TNF and UV, could be repressed in cyclophilin D-transfected cells, and TNF was more efficiently inhibited. Fig. 5C shows that the proapoptotic activity of arsenic trioxide could also be inhibited by cyclophilin D.

Cyclophilin D Inhibits Exogenous Apoptosis Inducers. The above data suggested that cyclophilin D acts as a specific inhibitor of the PT-pore. Because the PT-pore complex is implicated in many diverse proapoptotic signalings (42), we wanted to know whether, besides cotransfected genes, cyclophilin D could also reduce cell death by exogenously applied apoptosis stimuli. In contrast to specific genes, these agents can be expected to activate many diverse proapoptotic pathways in the cell. We chose TNF and UV irradiation because they induce apoptosis by different pathways that nevertheless activate the PT-pore (43, 44). In addition, we used arsenic trioxide, a therapeutic agent used in the treatment of acute promyelocytic leukemias that, although its mode of action is less known, also seems to activate the PT-pore (45). We performed these experiments in HeLa cells to assess how general the repression activities of cyclophilin D in other cell types are. Fig. 5 reveals that cyclophilin D was also active as an apoptosis repressor in HeLa cells. Both apoptosis inducers, TNF and UV, could be repressed in cyclophilin D-transfected cells, and TNF was more efficiently inhibited. Fig. 5C shows that the proapoptotic activity of arsenic trioxide could also be inhibited by cyclophilin D.
many genes that inhibit proapoptotic stimuli are up-regulated in tumors (46, 47). We have found cyclophilin D to repress apoptosis induction via the PT-pore, which is activated by many diverse stimuli. We therefore tested the expression level of cyclophilin D in a tumor expression profiling blot that covers a number of different human tumors. This membrane contains RNA of both normal and tumor tissue from each patient. Fig. 6 shows that cyclophilin D is specifically and significantly up-regulated in human tumors of the breast, uterus, and ovary. The other tested tumors displayed a normal distribution of the expression level of this gene.

**DISCUSSION**

Because the regulation of apoptosis is of profound relevance for a number of diseases, especially cancer, its molecular regulation is the focus of intense investigations. In this report we have further analyzed apoptosis induction by ANT-1, a component of the PT-pore that we found among genes from a screen for proapoptotic genes (16, 17, 29, 48, 49). Importantly, ANT-1 is up-regulated in the prevalent degenerative heart disease dilated cardiomyopathy (19), which is characterized by an excess of apoptotic myocytes early in the progression of this fatal disease (20). A multitude of data suggest that apoptosis is mediated by specific protein-protein interactions. Consequently, both repressors and inducers exhibit their activity on overexpression. Using this correlation, our molecular analysis of ANT-1-induced apoptosis led us to its interactor, cyclophilin D, which is able to repress ANT-1-induced cell death. Additional studies indicated that cyclophilin D inhibited the activation of the PT-pore by many other proapoptotic stimuli. In this study we have also demonstrated that cyclophilin D is significantly up-regulated in various tumors of reproductive tissues (Fig. 6). Consequently, starting with a gene involved in a degenerative heart disease (ANT-1), our studies proceeded to a gene that seems to be involved in a proliferative disease (cyclophilin D). We have therefore collected evidence that the PT-pore contains at least two proteins relevant for diseases.

We propose that cyclophilin D represents a new kind of apoptosis inhibitor that is effective at a functionally different level than the known inhibitors of the Bcl-2 family. First, the antiapoptotic activity of cyclophilin D does not correlate with the established cell death inhibitors Bcl-2 and Bcl-XL (Fig. 3, B and C). Furthermore, whereas the Bcl-2 proteins can also function independently of the PT-pore (7), cyclophilin D appears to be specific for this protein complex (see below). Lastly, cyclophilin D interacts with ANT-1, a component of the mitochondrial inner membrane, and is therefore also locally separated from the Bcl-2 system. Given the involvement of ANT-1 in dilated cardiomyopathy and cyclophilin D in tumors, our findings could therefore have a therapeutic value.

We suggest that cyclophilin D is specific for the PT-pore in its activity to repress apoptosis. Several lines of evidence support this view. First, cyclophilin D displays the same cell death repression profile as BA, which is a known specific inhibitor of the PT-pore (Figs. 3 and 4). Secondly, cyclophilin D is a potent repressor of ANT-1-induced apoptosis, which seems to activate the PT-pore directly for apoptosis induction (17). Thirdly, cyclophilin D is a component of the PT-pore. To our knowledge, it has not been found in other protein complexes in the cell. It is therefore likely that cyclophilin D is confined in its activity to the PT-pore. In line with this, a cyclophilin D construct devoid of its mitochondrial import signal showed a dramatic reduction of apoptosis repression (data not shown).

Furthermore, the cell death repression is independent of its peptidyl prolyl isomerase activity (Fig. 2C). It is therefore likely that permanent interactions, such as those with components of the PT-pore [especially the strong association with ANT-1 (50)], and not transient protein contacts, are relevant for its antiapoptotic function. How would the interactions that allow cyclophilin D to act as a repressor for apoptosis induction appear? The PT-pore is converted into a nonspecific channel on apoptosis induction (42). Consequently, cyclophilin D could act to close the PT-pore channel and only allows a controlled exchange of ADP and ATP. This model would require that cyclophilin D is inactivated during cell death. In fact, a recent paper noted that cyclophilin D mRNA is degraded on apoptosis induction (51). This could be one mechanism (but not necessary the only one) for the inactivation of cyclophilin D. Other, additional mechanisms could be conformational changes or the recruitment of additional binding partners.

In former models, cyclophilin D has been supposed to act in a proapoptotic way because cyclosporin that binds and inhibits cyclophilins can also inhibit PT-pore activation and apoptosis induction (42, 52, 53). However, because the cell contains at least eight different cyclophilins outside of the mitochondria, the specificity of the cyclosporin effect on the PT-pore has been questioned (54). In line with this, we observed that cyclosporin A could reduce ANT-1-induced apoptosis in 293T cells by only 20% under the best conditions (data not shown), suggesting that this compound acts differently from cyclophilin D. In addition, there is accumulating evidence that demonstrates that cyclosporin can also induce apoptosis (55–57). Furthermore, the inhibitory function of cyclosporins is based on the ability of cyclophilins to act as isomerases (52, 53). Because we have found that the antiapoptotic function of cyclophilin D is not dependent on its
peptidylprolyl isomerase activity activity, other effects must be responsible for the inhibitory or inducing effects of cyclosporin A. In contrast to our study, a recent report showed that cyclophilin D can function as a repressor for apoptosis only when it is functional as a peptidylprolyl isomerase (58). However, in these experiments, only the potential of the inner membrane of mitochondria was taken as an indicator for apoptosis, which is fraught with potential artifacts (1) and does not always correlate with cell death induction (7, 59). When apoptosis was measured by membrane permeability, these authors observed a temporal delay of only 2 h by cyclophilin D expression. We obtained much stronger effects using the degradation of genomic DNA as an established parameter for apoptosis. Our finding that cyclophilin D has an activity independent of its peptidylprolyl isomerase (Fig. 2C) is not without precedent: cyclophilin A can mediate HIV replication by interacting with Gag without its isomerase function (60), and the porcine kidney peptidylprolyl isomerase can fold the creatine kinase without its catalytic activity (61).

Given the possible involvement of ANT-1 in the heart disease dilated cardiomyopathy (17, 19), we investigated apoptosis by ANT-1. Although this ADP/ATP transporter can be regarded as an indirect functional component of the respiratory chain, this protein complex seems to be dispensable for cell death (Fig. 1C). Our results also show that the PT-pore components ANT-1 and Bax activate cell death differently (5, 6) because the antiapoptotic Bcl-XL could distinguish between these apoptosis inducers (Fig. 3). Furthermore, BA and cyclophilin D have differing effectiveness in reducing the effects of these proapoptotic genes (Fig. 3). We also found that specific sequences are required for ANT-1 to induce apoptosis (Fig. 1B).

According to a proposed structure of ANT-1 (26), the domains responsible for apoptosis induction lie on both sides of the inner mitochondrial membrane: whereas the third loop protrudes into the matrix; the NH₂ terminus and the second and fourth loop face the intermembrane space. Consequently, assuming that cyclophilin D resides in the matrix of mitochondria, only the third loop that faces this compartment could be bound and inhibited by cyclophilin D. However, cyclophilin D has also been found in the innermembrane space and could therefore contribute to the repression of these apoptosis-inducing domains of ANT-1 (32). An additional putative inhibitor of ANT-1 is the creatine kinase that is also located in the innermembrane space. Of note, this molecule could likewise, but less efficiently, reduce ANT-1-mediated cell death (Fig. 2A), and its ligand, creatine, reduces apoptosis in many different diseases ranging from Alzheimer’s disease to amyotrophic lateral sclerosis (62–64). This underscores the validity of our approach with cotransfected PT-pore components. Because the PT-pore is supposed to consist of additional proteins (4), other, previously unknown constituents could also act as inhibitors of ANT-1.

We have found that cyclophilin D is significantly increased in tumors of the breast, uterus, and ovary (Fig. 6). If the PT-pore is involved in so many kinds of apoptosis induction by being seemingly activated by a multitude of different apoptosis inducers (42), why is cyclophilin D only up-regulated in tumors associated with reproductive tissues in females? Whereas a definitive answer cannot be given at this time, these tissues are estrogen dependent. It is known that apoptosis repression is especially important for tumorigenesis of such tissues (65, 66). There is also a growing body of evidence that
cytochrome c play a role in the development of tumors (67–69). Consequently, it is possible that cyclophilin D is involved in apoptosis repression and hence in the growth and progression of hormone-dependent tumors.

Expression of proapoptotic genes allows induction of specific signals in the cell for apoptosis induction. Not all components of the PT-pore are active for apoptosis induction or repression (Figs. 1A and 2A). This stresses the specificity of the protein-protein contacts that are responsible for cell death induction. In addition, this could also indicate that some of the PT-pore components might not be involved in apoptosis regulation. After all, the PT-pore has the important function in healthy cells of transporting metabolic intermediates in and out of the mitochondria. Whereas the role of the PT-pore itself in apoptosis induction has firmly been established, the contribution of the various components has been less clear. We have demonstrated here that the PT-pore contains both repressors and inducers of apoptosis, both of which are relevant for specific diseases.

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