

Mitochondrial Control of Nuclear Apoptosis

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Summary

Anucleate cells can be induced to undergo programmed cell death (PCD), indicating the existence of a cytoplasmic PCD pathway that functions independently from the nucleus. Cytoplasmic structures including mitochondria have been shown to participate in the control of apoptotic nuclear disintegration. Before cells exhibit common signs of nuclear apoptosis (chromatin condensation and endonuclease-mediated DNA fragmentation), they undergo a reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$) that may be due to the opening of mitochondrial permeability transition (PT) pores. Here, we present direct evidence indicating that mitochondrial PT constitutes a critical early event of the apoptotic process. In a cell-free system combining purified mitochondria and nuclei, mitochondria undergoing PT suffice to induce chromatin condensation and DNA fragmentation. Induction of PT by pharmacological agents augments the apoptosis-inducing potential of mitochondria. In contrast, prevention of PT by pharmacological agents impedes nuclear apoptosis, both in vitro and in vivo. Mitochondria from hepatocytes or lymphoid cells undergoing apoptosis, but not those from normal cells, induce the disintegration of isolated Hela nuclei. A specific ligand of the mitochondrial adenine nucleotide translocator (ANT), bongkreikic acid, inhibits PT and reduces apoptosis induction by mitochondria in a cell-free system. Moreover, it inhibits the induction of apoptosis in intact cells. Several pieces of evidence suggest that the proto-oncogene product Bcl-2 inhibits apoptosis by preventing mitochondrial PT. First, to inhibit nuclear apoptosis, Bcl-2 must be localized in mitochondrial but not in nuclear membranes. Second, transfection-enforced hyperexpression of Bcl-2 directly abolishes the induction of mitochondrial PT in response to a protonophore, a pro-oxidant, as well as to the ANT ligand atractyloside, correlating with its apoptosis-inhibitory effect. In conclusion, mitochondrial PT appears to be a critical step of the apoptotic cascade.

Since it has been shown that anucleate cells (cytoblasts) can be induced to undergo programmed cell death (PCD)¹ (1–3), it has become clear that a cytoplasmic PCD pathway must function independently from the nucleus. Both mitochondria (4) and specific ced-3-like proteases

(5–7) have been accused of participating in the cytoplasmic control of apoptotic nuclear disintegration. We and others (8–12) have recently demonstrated that cells undergo a reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$) before they exhibit common signs of nuclear apoptosis (chromatin condensation and endonuclease-mediated DNA fragmentation). This applies to different cell types (neurons, fibroblasts, B and T lymphocytes, pre-B cells and thymocytes, myelomonocytic cells) and to different physiological apoptosis inducers (growth factor withdrawal, tumor necrosis factor, ceramide, glucocorticoids, activation-induced cell death, positive and negative selection, irradiation; 8–13). Moreover, these observations extend to pathogen-induced apoptosis, including irradiation-induced PCD (13) and HIV-1-triggered T lymphocyte PCD (14). When PCD is prevented either by genetic manipulations (e.g., p53 loss mutation, bcl-2 hyperexpression) or by pharmacological

¹Abbreviations used in this paper: ANT, adenine nucleotide translocator; Atr, atractyloside; BA, bongkreikic acid; CsA, cyclosporin A; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DEX, dexamethasone; diamide, diazenedicarboxylic acid bis 5*N,N*-dimethylamide; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; $\Delta\Psi_m$, mitochondrial transmembrane potential; GalN, D-galactosamine; ICE, IL-1 β converting enzyme; MCB, monochlorobimane; mCICCP, carbonyl cyanide m-chlorophenylhydrazone; mtDNA, mitochondrial DNA; PCD, programmed cell death; PT, permeability transition; ROS, reactive oxygen species; RR, ruthenium red; *ter*-BHP, *ter*-butylhydroperoxide.

N. Zamzami and S.A. Susin contributed equally to this paper. This paper is dedicated to José Uriel.

agents (*N*-acetylcysteine, protease inhibitors, linomide), both mitochondrial and nuclear signs of apoptosis are abolished (10, 12, 13). Moreover, cells that have lost their $\Delta\Psi_m$ appear to be irreversibly programmed to die (10). Although these observations suggest the involvement of mitochondria in apoptosis, they do not clarify the cause-effect relationship between mitochondrial dysfunction and subsequent nuclear apoptosis. It appears clear that reactive oxygen species (ROS), which may be generated by uncoupled mitochondria (9, 12), are not essential for the apoptotic process (15–17). Thus, whenever a cause-effect relationship between mitochondrial disorders and nuclear apoptosis exists, it must be mediated by factors other than ROS. The aim of this paper was to unravel the existence of such a pathway linking mitochondrial dysfunction to nuclear disintegration.

As to the mechanism of apoptotic $\Delta\Psi_m$ disruption, pharmacological experiments suggest that it involves the opening of so-called mitochondrial permeability transition (PT) pores (12, 18). Under normal conditions, the inner mitochondrial membrane is quasi-impermeable for small molecules, thus allowing for the creation of the electrochemical gradient which is indispensable for mitochondrial function. However, in determined circumstances, opening of PT pores or “megachannels” allows for the free distribution of solutes of <1,500 daltons and of some proteins, thereby disrupting the $\Delta\Psi_m$ and associated mitochondrial functions (19, 20). In isolated mitochondria, PT is accompanied by colloid osmotic swelling and uncoupling of oxidative phosphorylation, as well as by the loss of low molecular weight matrix molecules such as calcium and glutathione (19–21). It may be important to note that PT is modulated by multiple different physiological and pharmacological inducers and inhibitors (for a review see reference 22) and that PT is both the cause and the consequence of $\Delta\Psi_m$ dissipation, as well as of reactive oxygen metabolite production (19–29). In other terms, PT results *ipso facto* in $\Delta\Psi_m$ disruption and later in ROS hyperproduction, but $\Delta\Psi_m$ reduction and ROS themselves can also provoke PT, as do many other factors (divalent cations, pH variations, peptides, etc; 22).

The exact molecular composition of the PT pore is not known. However, it appears that at least one inner mitochondrial transmembrane protein, namely the adenine nucleotide translocator (ANT), is involved in PT pore formation (for reviews see references 19, 20) and that ANT associates with several molecules of the outer mitochondrial membrane such as the peripheral benzodiazepine receptor and the voltage-dependent anion channel (30). ANT ligands such as atractyloside (Atr) and bongkrekic acid (BA) enhance or reduce the probability of PT, respectively (31–35).

Based on these premises, we have tested the hypothesis that PT might be the critical event determining the apoptosis-inducing potential of mitochondria. Using a cell- and cytosol-free system in which purified mitochondria and nuclei are confronted, we show that induction of PT by the ANT ligand Atr or other less specific PT inducers causes isolated mitochondria to trigger nuclear apoptosis. In contrast, inhibition of PT by the Atr antagonist BA, as well

as by a variety of additional PT inhibitors, abolishes mitochondria-mediated nuclear apoptosis. The apoptosis-inhibitory proto-oncogene product Bcl-2 functions as an endogenous inhibitor of mitochondrial PT. These data establish mitochondrial PT as a critical event of apoptosis.

Materials and Methods

Animals and In Vivo Treatments. Male 6–10-wk-old BALB/c mice were injected simultaneously with D-galactosamine (GalN; 10 mg i.p.) and/or LPS from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO; 50 μ g i.v.), 5 h before removal of the liver (36). Alternatively, splenocytes were recovered from BALB/c mice 12 h after injection of 1 mg i.p. dexamethasone (DEX; Sigma Chemical Co.) in 200 μ l PBS or PBS alone (10, 37).

Cell Lines and In Vitro Culture Conditions. U937 cells were depleted from mitochondrial DNA (mtDNA) by continuous ethidium bromide selection for 4 mo (15). Control experiments revealed that such cells become resistant to antimycin A, which blocks the mtDNA-encoded complex III. Moreover, no mtDNA could be detected by PCR (not shown). 2B4.11 T cell hybridoma cell lines stably transfected with an SFVV.neo vector containing the human *bcl-2* gene or the neomycin (Neo) resistance gene only (38, 39) were kindly provided by Jonathan Ashwell (National Institutes of Health, Bethesda, MD). Cells were cultured in RPMI-1640 medium containing 5% FCS. Apoptosis was induced by culturing cells in the presence of the indicated concentration of diazenedicarboxylic acid bis 5*N,N*-dimethylamide (diamide) or carbonyl cyanide *m*-chlorophenylhydrazone (mCICCP; both from Sigma Chemical Co.). DNA fragmentation of non- or γ -irradiated (10 Gy) thymocytes (10^6 cells/lane) was monitored after culturing cells for 4 h in the presence of DEX (1 μ M), etoposide (10 μ M; Sigma Chemical Co.), and/or BA (50 μ M; purified as described in reference 40), kindly provided by Dr. J.A. Duine (Delft University, Delft, The Netherlands).

Cell-free System of Apoptosis. Nuclei from HeLa or 2B4.11 cells were purified on a sucrose gradient, as described (41), and were resuspended in CFS buffer (220 nM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM $\text{PO}_4\text{H}_2\text{K}$, 0.5 mM EGTA, 2 mM Cl_2Mg , 5 mM pyruvate, 0.1 mM PMSF, 2 mM ATP, 50 μ g/ml creatine phosphokinase, 10 mM phosphocreatine, 1 mM dithiothreitol, and 10 mM HEPES-NaOH, pH 7.4; reagents from Sigma Chemical Co.). Nuclei were conserved at -20°C in 50% glycerol for up to 8 d as described (41, 42). Mitochondria were purified from BALB/c mouse livers, splenocytes, or U937 cells on a Percoll gradient (43) and were stored on ice in B buffer (400 mM mannitol, 10 mM $\text{PO}_4\text{H}_2\text{K}$, 5 mg/ml BSA, and 50 mM Tris-HCl, pH 7.2) for up to 4 h. For quantitation of nuclear apoptosis, both nuclei (5,000 g, 5 min) and mitochondria (2×10^4 g, 3 min) were spun down and washed twice in CFS buffer before being mixed. In standard conditions, mitochondria (500 ng/ μ l protein final concentration) were cultured at 37°C for 90 min with 10^3 nuclei per μ l CFS containing a number of different agents: AIF₃ (20 μ M), Atr (5 mM; Sigma Chemical Co.), BA (50 μ M), CaCl_2 (500 μ M), mCICCP (10 μ M), diamide (100 μ M), cyclosporin A (CsA, 10 μ M; Sandoz AG, Basel, Switzerland), *N*-methylVal-4-CsA (SDZ 220-384, 10 μ M; kindly provided by Dr. Roland Wenger, Sandoz), monochlorobimane (MCB; 30 μ M), phosphotyrosine (P; 10 mM), ruthenium red (RR, 100 μ M; Sigma Chemical Co.), *tert*-butylhydroperoxide (*tert*-BHP, 50 μ M; Sigma Chemical Co.), ZnCl_2 (1 mM), AcYVAD-CHO (IL-1 β converting enzyme [ICE] inhibitor I), AcYVAD-chloromethylketone (ICE inhibitor II), and/or AcDEVD-CHO (inhibitor of CPP32/

Ced3/Yama; Bachem, Basel, Switzerland). Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 μ M) and examined by fluorescence microscopy (5), or were analyzed by agarose gel electrophoresis (10⁶ nuclei/lane) (44).

Cytofluorometric Analysis. For $\Delta\Psi_m$ determinations, isolated mitochondria were incubated for 15 min at 37°C in the presence of DiOC₆(3) (80 nM) (45), followed by addition of mCICCP (50 μ M), BA (50 μ M) and/or Atr (5 mM), and recording of the fluorescence in an Elite cytofluorometer (Coulter Corp., Hialeah, FL) 5 min later. Loss of nuclear DNA (hypoploidy) was determined by propidium iodide staining of ethanol-fixed cells, as described (46).

Large Amplitude Swelling of Isolated Mitochondria. Large amplitude swelling is a colloidosmotic process that is observed among isolated mitochondria undergoing PT in solutions containing low protein concentrations (22). For determination of swelling, mitochondria were washed and resuspended in B buffer (100 μ g protein/10 μ l buffer), followed by addition of 90 μ M CFS buffer and recording of adsorption at 540 nm in a spectrophotometer (model DU 7400; Beckman Instruments, Inc., Fullerton, CA), as described (26). The loss of absorption induced by 5 mM Atr within 5 min was considered 100% the value of large amplitude swelling.

Characterization of Factors Contained in the Supernatant of Mitochondria. Hepatic mitochondria (1 mg/ml in CFS buffer) were left untreated or were incubated with Atr (5 mM) for 10 min at room temperature, followed by ultracentrifugation (1.5 \times 10⁵ g, 30 min, 4°C). Supernatants were either left untreated or centrifuged through a Centricon 10 membrane (Amicon Inc., Beverly, MA) to separate proteins with an approximate molecular mass of > and <10 kD, following the manufacturers' recommendation. The fraction >10 kD was reconstituted with CFS to the original volume. Supernatants (50% volume) were mixed with nuclei (10³/ μ l) in the presence or absence of various antioxidants (50

μ M *N*-*t*-butyl- α -phenylnitron, 230 μ M trolox, 600 μ M L-ascorbate, or 1 mg/ml catalase; Sigma Chemical Co.), and nuclei were stained with DAPI after 90 min of culture at 37°C.

Results and Discussion

Isolated Mitochondria Undergoing PT Induce Nuclear Apoptosis in a Cell-free System. One of the PT pore constituents is the ANT. The ANT adopts different molecular conformations when exposed to two specific ligands, Atr and BA (31, 47). Atr favors the opening of the PT pore, whereas BA reduces the probability of PT pore gating (31–35, 47). We have tested the capacity of purified mitochondria with open and closed PT pores to induce nuclear apoptosis in a modified cell-free system (4, 42). Mitochondria were purified from the murine liver and were mixed with HeLa nuclei in an isotonic buffer containing an ATP regeneration system. On their own, unmanipulated mitochondria derived from healthy cells are incapable of inducing signs of nuclear apoptosis such as chromatin condensation and endonuclease-mediated DNA fragmentation. However, mitochondria exposed to a dose of Atr that causes PT, determined either as large amplitude swelling (Fig. 1 A) or as disruption of the $\Delta\Psi_m$ (Fig 1 B), do induce nuclear apoptosis (Fig. 1 C) in a time- and dose-dependent fashion (Fig. 1 D). Atr does not induce apoptosis itself; it only favors the induction of nuclear apoptosis when mitochondria are present (Fig. 1 C). In the presence of BA, mitochondria fail to undergo PT in response to Atr (Fig. 1, A and B) and lose the capacity to induce chromatin condensation (Fig. 1 C) and associated oligonucleosomal DNA fragmentation (Fig.

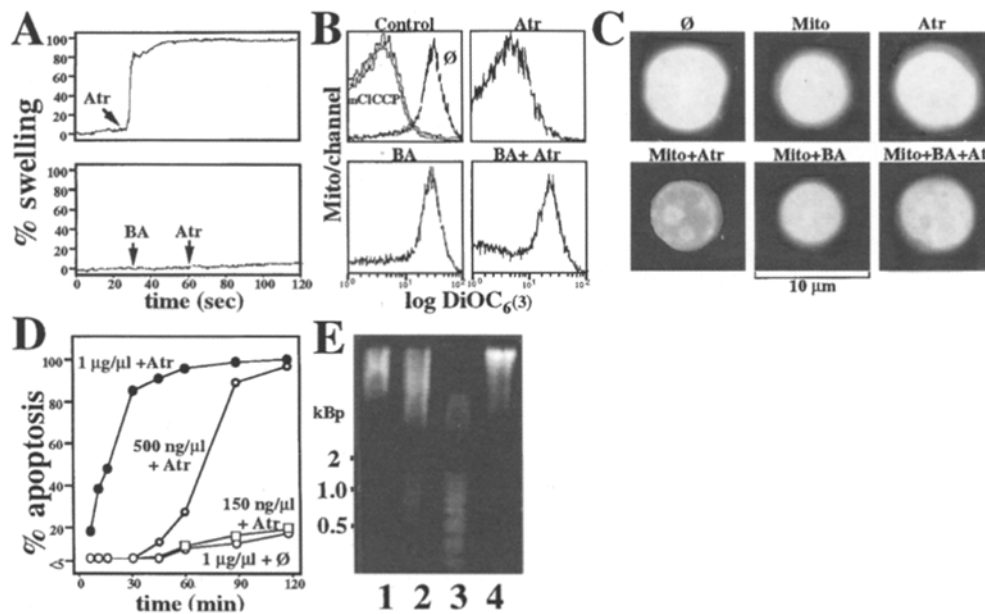


Figure 1. Regulation of mitochondrial PT and mitochondria-mediated nuclear apoptosis by two adenine nucleotide translocator ligands. (A) Effect of atractyloside (Atr) and bongkreikic acid (BA) on mitochondrial large amplitude swelling. (Arrows) Time points at which BA (50 μ M) and/or Atr (5 mM) were added to mitochondria. (B) $\Delta\Psi_m$ of Atr- and BA-treated mitochondria, as determined by incorporation of DiOC₆(3) into mitochondria, 5 min after addition of Atr and/or BA. Incubation with the protonophor mCICCP, which completely disrupts the $\Delta\Psi_m$, unravels DiOC₆(3) background fluorescence. (C) Chromatin distribution of isolated HeLa nuclei cultured with mitochondria, Atr, and/or BA for 90 min. Single nuclei stained with DAPI representing the dominant phenotype

(\geq 80%) are shown. (D) Time and dose dependence of nuclear chromatin condensation. Nuclei were incubated during the indicated interval with the specified amount of mitochondria (doses in μ g protein) and/or Atr. (E) Fragmentation of nuclear DNA induced by mitochondria. Purified HeLa nuclei were cocultured with mitochondria (500 ng/ μ l), Atr, and/or BA for 90 min (same conditions as in C), followed by agarose gel electrophoresis of ethidium bromide-stained DNA. Lane 1: Atr only; lane 2: mitochondria only; lane 3: mitochondria plus Atr; and lane 4: mitochondria plus Atr plus BA.

1 E). These findings suggest that PT controls the apoptosis-inducing capacity of mitochondria.

Mitochondria Lacking mtDNA Can Undergo PT and Cause Nuclear Apoptosis in a Cell-free System. Although most proteins contained in mitochondria are encoded by nuclear genes, a number of proteins including some components of the respiratory chain complexes I, III, and IV, are encoded by the mitochondrial genome. Previously, it has been reported that cell lines lacking mtDNA can undergo full-blown nuclear apoptosis (15), and this finding could give rise to the interpretation that mitochondria are not important for the control of apoptosis. To challenge this (over)interpretation, we purified mitochondria from cells lacking mtDNA (ρ^0 cells), as well as from control ρ^+ cells, and tested their apoptosis-inducing potential. As shown in Fig. 2 A, ρ^0 mitochondria can undergo large amplitude swelling in response to Atr, exactly as do ρ^+ control organelles. Moreover, in the presence of Atr, ρ^0 mitochondria are as efficient inducers of nuclear disintegration, as are control ρ^+ mitochondria (Fig. 2 B). This indicates that all mitochondrial functions critical for apoptosis induction are encoded by nuclear genes. Thus, in accord with the published data (15), the capacity of mitochondria to induce nuclear apoptosis does not depend on the presence of mtDNA. Furthermore, the fact that respiration-deficient ρ^0 mitochondria (which may be expected to produce less ROS than ρ^+ control organelles) conserve their proapoptotic activity, suggests that ROS do not mediate apoptosis in this cell-free system.

Strict Correlation Between PT-associated Swelling and Nuclear Apoptosis Induction. The above results suggest that mitochondria are indeed efficient inducers of nuclear apoptosis, provided that they are undergoing PT. This conclusion is corroborated by the strict correlation between PT and the proapoptotic effect of mitochondria, when PT is induced by a variety of different molecules: Atr, the pro-oxidant *ter*-BHP, and calcium ions via the thiol-cross-linking agent diamide or the protonophore mClCCP (Fig. 3). All these re-

agents are thought to act via distinct mechanisms: Atr by virtue of its capacity to interact with the ANT (31–35); calcium via conformational effects on proteins that are yet poorly understood (19, 20, 22); hydroperoxides via oxidation of mitochondrial glutathione and pyridine nucleotides (48); diamide via its thiol-cross-linking action on the ANT (49, 50); and protonophores via dissipation of the proton gradient ($\Delta\Psi_m$), then entailing PT as a secondary phenomenon (24, 27, 28). Control experiments indicate that none of these reagents induces nuclear apoptosis by itself, i.e., in the absence of mitochondria (not shown). Atr-induced swelling and nuclear apoptosis are efficiently inhibited by BA and CsA, as well as by the thiol reagent MCB. CsA can be substituted for by the nonimmunosuppressive CsA analogue *N*-methylVal-4-CsA (51), indicating that its PT and apoptosis-inhibitory effect is not mediated via calcineurin. A series of substances previously reported to inhibit apoptosis in a cell-free system (4) can also inhibit both PT and mitochondria-mediated nuclear apoptosis: phosphotyrosine, $ZnCl_2$, and AlF_3 (Fig. 3). Other substances (4) have no or little (<20%) inhibitory effects on Atr-induced swelling and apoptosis: calpain inhibitors I and II, GTP γ S, and ionomycin. Similarly, synthetic tetrapeptide inhibitors of ICE and of CPP32/Yama fail to interfere with PT and PT-dependent nuclear apoptosis (Table 1). Calcium-driven but not Atr-induced PT and apoptosis are selectively inhibited by RR, a specific inhibitor of the mitochondrial calcium uniport (52), underscoring the fact that proapoptotic calcium effects are indeed mediated by mitochondria. As expected (50), the syn-9,10-dioxo-bimanine halogen derivative MCB is particularly effective in inhibiting PT and apoptosis induced by diamide (Fig. 3). In accord with published data (for a review see reference 22), none of the inhibitors used in this study is capable of providing long-term (>30 min) protection against mitochondrial swelling in response to the whole panel of PT inducers. This probably reflects the profound differences in the molecular mechanisms of PT caused by different inducers (19, 20, 22, 24, 27, 28, 48–50).

In synthesis, the strict correlation existing between mitochondrial PT and mitochondria-mediated nuclear apoptosis suggests that PT is indeed a crucial event in the regulation of apoptosis induction by mitochondria. Moreover, the fact that none of the inhibitory substances (BA, CsA, MCB, RR, phosphotyrosine, $ZnCl_2$, AlF_3) suppresses PT and apoptosis in response to all PT inducers (Fig. 3) suggests that they do not directly affect nuclei but rather act via PT modulation. Finally, the data summarized in Fig. 3 underscore the complex pharmacology of mitochondrial PT.

Mitochondria from Cells Undergoing Apoptosis Transfer Nuclear Apoptosis to a Cell-free System. According to several studies (8–10, 12), mitochondrial function is perturbed early during the apoptotic process. Accordingly, mitochondria isolated from hepatocytes exposed *in vivo* to an apoptosis-inducing combination of GalN and LPS (36, 53), but not control cells treated with GalN or LPS only, display a reduced uptake of the cationic lipophilic dye 3,3' dihexyloxocarbocyanine iodide (DiOC₆[3]) (Fig. 4 A), indicating a

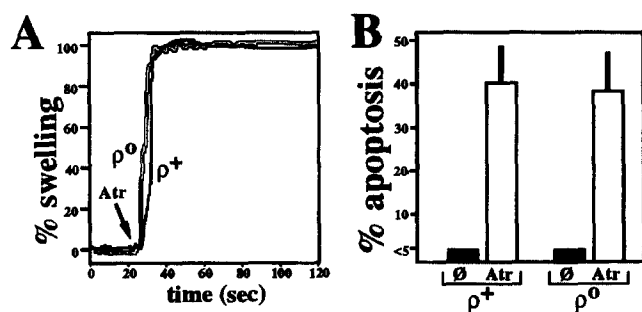


Figure 2. Mitochondrial swelling and nuclear apoptosis induction do not require mitochondrial DNA. (A) Large amplitude swelling of mitochondria lacking mtDNA. Mitochondria obtained from normal U937 cells (ρ^+) or U937 cells lacking mtDNA (ρ^0) were incubated while assessing absorbance at 540 nm. (Arrow) Addition of Atr (5 mM). (B) Induction of nuclear apoptosis by mitochondria lacking mtDNA. HeLa nuclei were cultured for 90 min with Atr and/or mitochondria obtained from ρ^+ or ρ^0 U937 cells, followed by determination of the frequency of apoptotic or damaged nuclei.

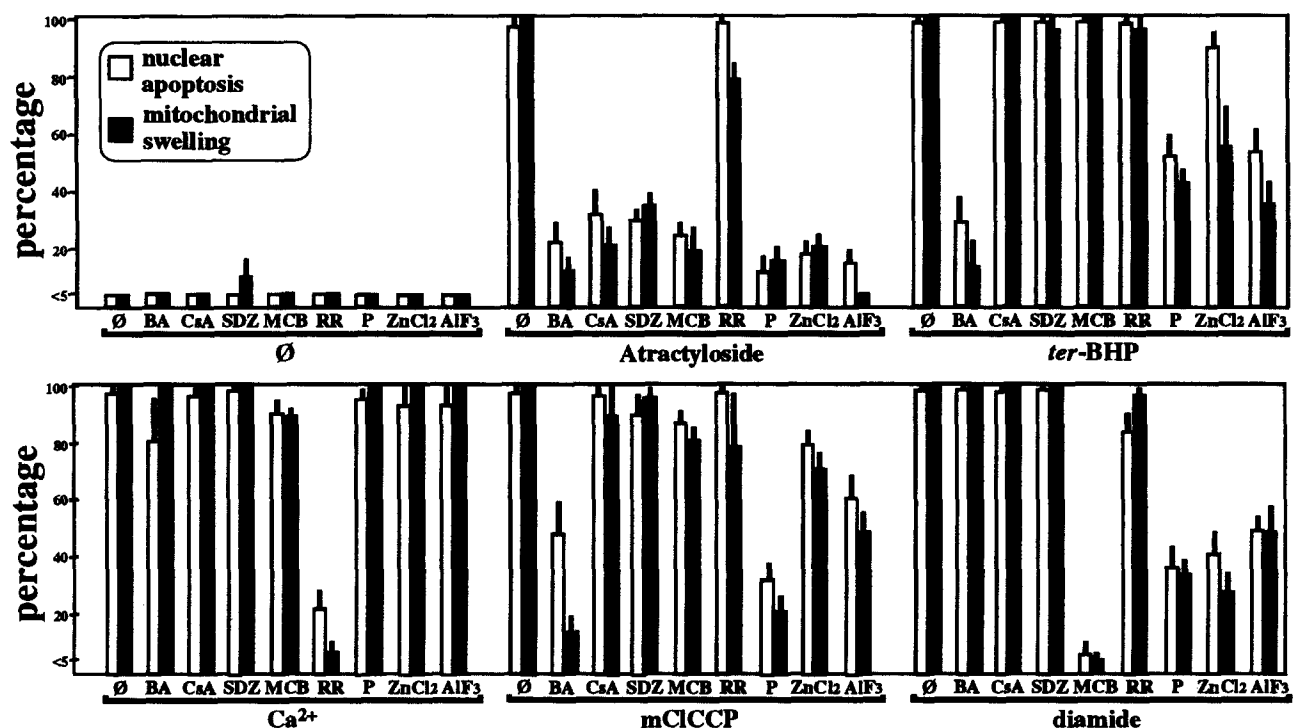


Figure 3. Correlation between mitochondrial swelling and mitochondrial induction of nuclear apoptosis. Mitochondria from hepatocytes were incubated with the PT inducers Atr (5 mM), *ter*-BHP (50 μ M), CaCl_2 (500 μ M), mClCCP (10 μ M), or diamide (100 μ M) and/or the PT inhibitors BA (50 μ M), CsA (10 μ M), *N*-methylVal-4-CsA (SDZ; 10 μ M), MCB (30 μ M), or RR (100 μ M). Mitochondria were also incubated with P (10 mM), ZnCl_2 (1 mM), or AlF_3 (20 μ M). The percentage of condensed nuclei cocultured with mitochondria was recorded after 90 min of incubation at 37°C (open columns, X \pm SEM of triplicates). Large amplitude swelling was recorded after 5–90 min of culture (black columns). Data are shown for 60 min, when the correlation between nuclear apoptosis and mitochondrial swelling is optimal. These results are representative of five independent experiments.

decrease of the $\Delta\Psi_m$. Such mitochondria from apoptotic liver cells cause nuclear apoptosis in vitro (Fig. 4 B). Similarly a fraction of mitochondria from splenocytes treated in vivo with the glucocorticoid analogue DEX (10, 37) display a reduced $\Delta\Psi_m$ (Fig. 5 A) and cause apoptosis of isolated Hela nuclei in vitro (Fig. 5 B). Thus, mitochondria from different cell types undergoing apoptosis in vivo are

endowed with the capacity of apoptosis induction in a cell-free system.

Inhibition of PT Inhibits Nuclear Apoptosis both In Vitro and In Vivo. The ANT ligand BA is the agent with the broadest PT-inhibitory spectrum among all substances tested thus far (Figs. 1 and 3) and is the only PT inhibitor that is truly specific for a mitochondrial structure. We

Table 1. Substances that Fail to Modulate PT and Mitochondria-dependent Nuclear Apoptosis

Substance	Dose range	Inhibition of PT	Inhibition of nuclear apoptosis
Calpain inhibitor I	100 μ M–1 mM	None*	None
Calpain inhibitor II	100 μ M–1 mM	None	None
ICE inhibitor I	100 μ M–500 μ M	None	None
ICE inhibitor II	100 μ M–500 μ M	None	None
AcDEVD-CHO	100 μ M–500 μ M	None	None
GTP γ S	100 μ M–1 mM	None	None
Ionomycin	10 μ M–100 μ M	None	None

*The indicated substances were employed to modulate the induction of PT in isolated mitochondria induced by the following reagents: Atr, *ter*-BHP, calcium, m-ClCCP, and diamide (same conditions and concentrations as in Fig. 3). Absence of inhibition indicates <20% suppression of either mitochondrial swelling (measured at 60 min as in Fig. 3) or nuclear condensation (measured at 90 min in the same conditions as in Fig. 3), in response to all tested PT inducers.

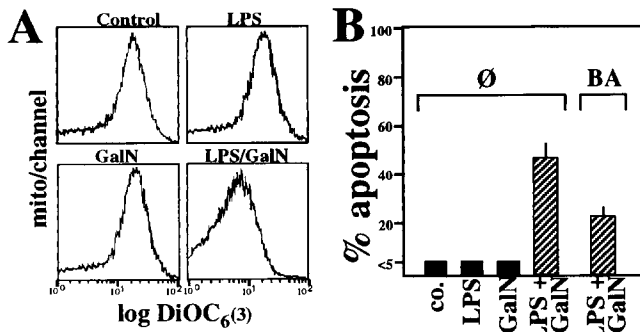


Figure 4. Mitochondria from apoptotic hepatocytes cause nuclear apoptosis in a cell-free system. (A) Reduction of the $\Delta\Psi_m$ in mitochondria from cells undergoing apoptosis in vivo. Liver mitochondria were obtained from animals treated with an apoptosis-inducing combination of GalN and/or LPS, followed by DiOC₆(3) labeling for $\Delta\Psi_m$ assessment. (B) Mitochondria from apoptotic hepatocytes induce nuclear apoptosis. After the indicated in vivo treatment, liver mitochondria were purified and added to HeLa nuclei in the presence or absence of BA (50 μ M), followed by evaluation of chromatin condensation as in Fig. 1. Data are representative of two independent experiments.

therefore tested the effect of BA on the mitochondria-mediated transfer of apoptosis from whole cells undergoing PCD to the cell-free system. As shown in Figs. 4 A and 5 A, BA partially reduces the proapoptotic effect of mitochondria from GalN/LPS-stimulated hepatocytes of DEX-primed splenocytes in vitro. This inhibition is significant: $48 \pm 10\%$ for GalN/LPS-treated hepatocyte mitochondria and $44 \pm 9\%$ for DEX-primed splenocyte mitochondria. In addition, BA is highly efficient ($>90\%$ inhibition) in preventing the death of intact thymocytes exposed to a series of different apoptosis inducers: DEX, irradiation, and topoisomerase inhibition (Fig. 6). These results corroborate the notion that mitochondria are indeed involved in the

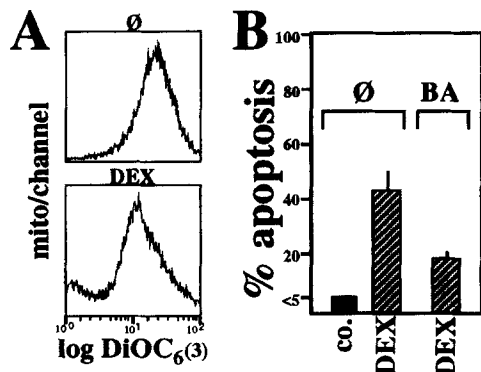


Figure 5. Transfer of apoptosis by mitochondria from apoptotic splenocytes. (A) $\Delta\Psi_m$ disruption of mitochondria from DEX-primed splenocytes. Mitochondria were purified from control splenocytes (co.) or from splenocytes exposed to the glucocorticoid DEX in vivo, followed by staining with DiOC₆(3). (B) Mitochondria from apoptotic splenic lymphocytes induce nuclear apoptosis. Mitochondria from control or DEX-primed splenocytes were mixed with HeLa nuclei either in the absence or in the presence of BA to assess their apoptosis-inducing potential as in Fig. 4 B. Splenic mitochondria were tested at a concentration of 250 ng/ μ l. Typical results out of three independent experiments are shown.

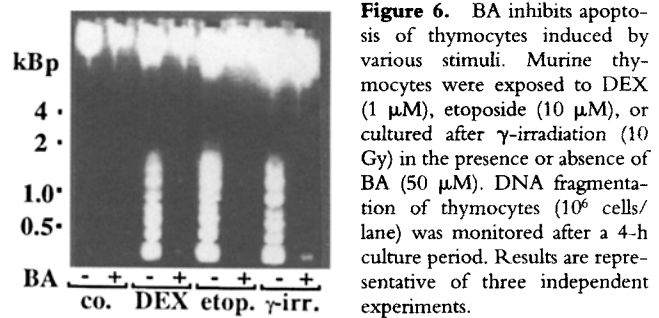


Figure 6. BA inhibits apoptosis of thymocytes induced by various stimuli. Murine thymocytes were exposed to DEX (1 μ M), etoposide (10 μ M), or cultured after γ -irradiation (10 Gy) in the presence or absence of BA (50 μ M). DNA fragmentation of thymocytes (10^6 cells/lane) was monitored after a 4-h culture period. Results are representative of three independent experiments.

apoptotic cascade in vivo and that mitochondrial PT is both sufficient and necessary to induce nuclear apoptosis.

Bcl-2 Inhibits Apoptosis by Preventing Mitochondrial PT. The proto-oncogene product Bcl-2 inhibits apoptosis in response to a number of different stimuli (for a review see reference 54) and prevents both the mitochondrial and the nuclear manifestations of apoptosis (12). Bcl-2 is localized in the mitochondrial outer membrane and endoplasmic reticulum, as well as in nuclear membranes (55–57). Within the mitochondrion, it is found at the inner-outer membrane contact site, where PT pores are expected to form (20). To map the antiapoptotic function of Bcl-2 either to mitochondria or to nuclei, we purified these organelles from hBcl-2-transfected murine T cell hybridoma cells (39), as well as from mock-transfected controls. Reconstitution experiments indicate that Atr-treated mitochondria from hBcl-2-transfected cells fail to provoke nuclear apoptosis (Fig. 7 A) in conditions in which mitochondria from vector-transfected cells (Fig. 7 A) or from hepatocytes constitutively lacking Bcl-2 expression (Figs. 1 and 3) do induce nuclear apoptosis. In contrast, nuclei from hBcl-2-transfected cells readily condense and fragment in the presence of Atr and control mitochondria (Fig. 7, A and B). Thus, in accord with previous genetic (55, 57) and functional (4) studies, the mitochondrial but not the nuclear localization of Bcl-2 is critical for its antiapoptotic function. In control experiments, mixtures of Bcl-2-transfected and control mitochondria induce apoptosis (Fig. 7 A), indicating that the Bcl-2-mediated inhibition of apoptosis acts in *cis* and cannot be attributed to cytosolic Bcl-2 contaminating the mitochondrial preparation. In addition, isolated mitochondria from Bcl-2-transfected cells are protected against Atr-induced PT, i.e., they fail to undergo large amplitude swelling and $\Delta\Psi_m$ disruption in response to Atr (Fig. 7, C and D). Bcl-2 is a potent inhibitor of some death pathways, including pro-oxidants (58), but is comparatively inefficient in preventing calcium-induced and antigen receptor-mediated PCD (38, 39, 59). We therefore tested whether Bcl-2 would be a universal inhibitor of PT or rather, whether it would have a selective effect. As shown in Fig. 8 A, Bcl-2 prevents large amplitude swelling of isolated mitochondria in response to M-CICCP and *ter*-BHP, but not in response to calcium or diamide. These data underscore that different PT inducers obey different mechanisms; this is also suggested by experiments involving PT inhibitors

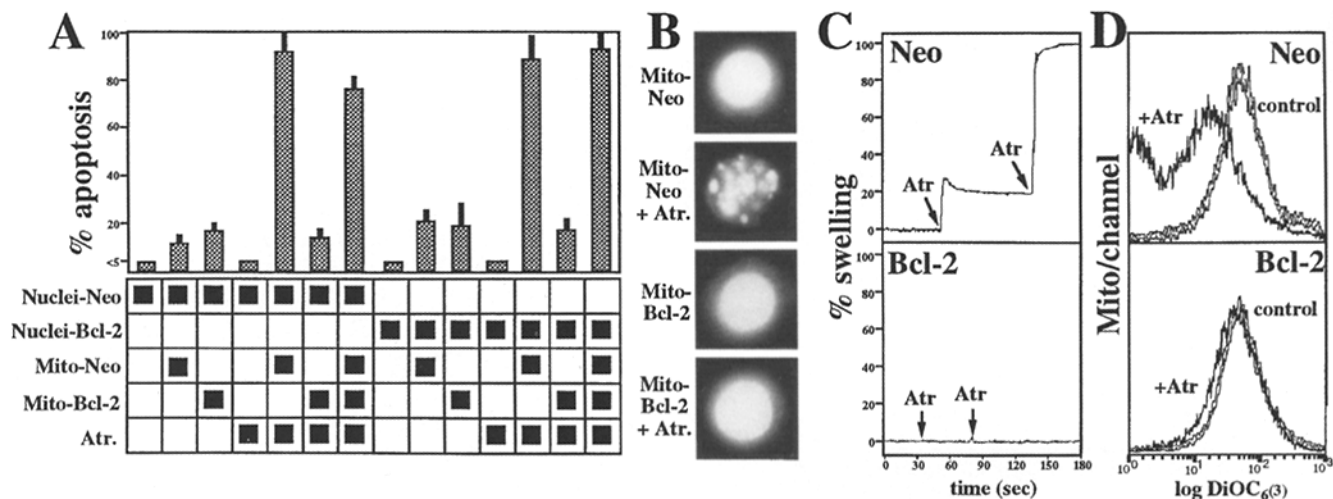


Figure 7. Mechanism of the antiapoptotic effect of Bcl-2. (A) Functional mapping of the site at which Bcl-2 acts to prevent Atr-induced apoptosis. Nuclei and mitochondria from Bcl-2- or Neo-transfected cells were cocultured in the presence or absence of Atr (5 mM), as indicated by black squares. After 90 min of coculture, nuclei were stained with DAPI and analyzed for apoptotic morphology. (B) Representative nuclei from Bcl-2-transfected cells incubated with the indicated type of mitochondria and/or Atr (same experiment as A). (C) Bcl-2 directly inhibits the Atr-induced large amplitude swelling of mitochondria. Mitochondria from Neo- or Bcl-2-transfected cells were monitored for large amplitude swelling (as in Fig. 1 A). (Arrows) Repeated addition of 2.5 mM Atr (final concentration 5 mM). (D) Bcl-2 inhibits the Atr-induced disruption of the mitochondrial transmembrane potential. Mitochondria were labeled with DiOC₆(3), cultured for 5 min in the presence or absence of 5 mM Atr, and were then analyzed by cytofluorometry.

(Fig. 3). The pattern of the bcl-2 effect corresponds most closely to that of BA, i.e., it inhibits PT induced by Atr (Figs. 3 and 7), m-ClCCCP, and *ter*-BHP, but not calcium or diamide (Figs. 3 and 8). Again, as in the case of BA, Bcl-2-mediated inhibition of PT results in the abolition of the apoptotic potential of isolated mitochondria. More importantly, the Bcl-2-driven inhibition of mitochondrial swelling (Fig. 8 A) correlates with its apoptosis-inhibitory potential in cells. Bcl-2 protects against apoptosis of T cell hybridoma cells induced by m-ClCCCP (Fig. 8 B) and oxidants such as H₂O₂ (58), yet fails to confer protection against diamide (Fig. 8 B) and CD3 cross-linking (12, 38,

39). Thus, Bcl-2 does not prevent apoptosis when death is induced via such agents as diamide (Fig. 8 B) against whose PT-inducing potential it does not protect (Fig. 8 A). Again, these data are in accord with the hypothesis that Bcl-2 prevents apoptosis by virtue of its PT-inhibitory potential.

A Soluble Factor Released from Mitochondria Undergoing PT Mediates Nuclear Disintegration. As shown above, mitochondria undergoing PT induce apoptotic nuclear disintegration in a cell-free system. Whereas some authors have shown that mitochondria are necessary to induce apoptosis in cell-free systems (4, 60), others have found that cytosolic (organelle-free) extracts may be sufficient to induce nuclear

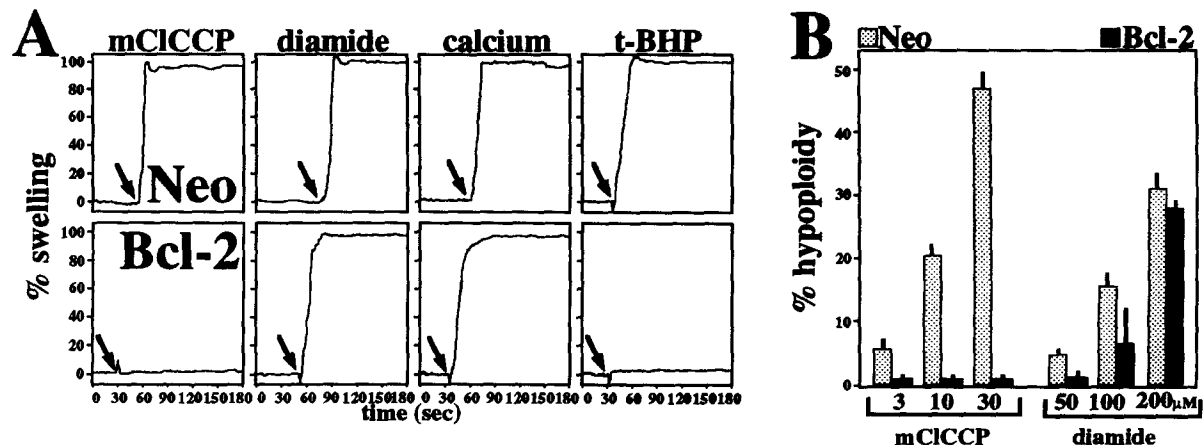


Figure 8. Correlation of the antiapoptotic and the PT-inhibitory effect of Bcl-2. (A) Effect of Bcl-2 on large amplitude swelling of isolated mitochondria. (Arrows) The indicated reagents were added (same concentrations as in Fig. 3), while absorbance at 540 nm was monitored. (B) Spectrum of Bcl-2-mediated inhibition of apoptosis in whole cells. Bcl-2, or Neo-transfected T cell hybridoma cells were cultured with the indicated dose of mClCCCP or diamide for 6 or 24 h, respectively. The percentage of cells with nuclear hypoploidy was determined after ethanol fixation and staining with propidium iodide.

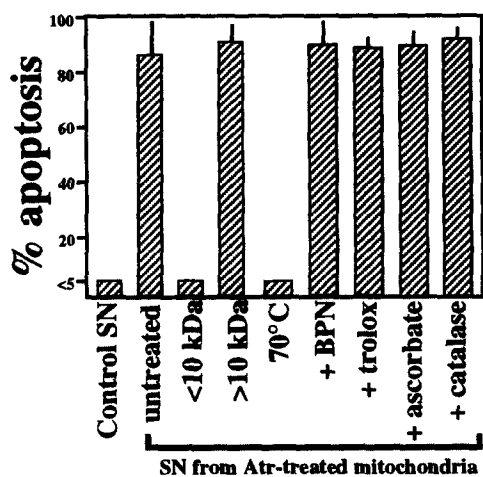


Figure 9. Partial characterization of a proapoptotic activity released from Atr-treated mitochondria. Liver mitochondria were incubated in the presence or absence of 5 mM Atr, followed by ultracentrifugation (150,000 g, 30 min). Isolated HeLa nuclei were incubated in the presence of this supernatant to determine the frequency of cells exhibiting apoptotic morphology (90 min, 37°C). Supernatants were heat treated (70°C, 5 min) or centrifuged through membranes with a molecular mass exclusion of ~10 kD before the test. Alternatively, the antioxidants *N-t*-butyl- α -phenylnitron (50 μ M), trolox (230 μ M), L-ascorbate (600 μ M), or catalase (1 mg/ml) were added to the assay.

apoptosis in vitro (5, 42). Prompted by this apparent contradiction, we tested whether mitochondria undergoing PT would release a soluble proapoptotic factor. As shown in Fig. 9, mitochondria treated with Atr release (a) soluble factor(s) into the supernatant (150,000 g, 30 min) that can induce chromatin condensation in isolated HeLa nuclei. This activity is heat sensitive (70°C, 5 min), has a molecular mass >10 kD, and is not neutralized by antioxidants such as *N-t*-butyl- α -phenylnitron or the water-soluble vitamin E analogue trolox (Fig. 9). In conclusion, at least part of the apoptotic activity of mitochondria is mediated by one or several proteins and does not involve ROS. PT-dependent release of proteins from mitochondria has been reported previously (61).

Concluding Remarks

As shown in this article, mitochondria from hepatic, myelomonocytic, or lymphoid cells induce nuclear apoptosis, provided that they undergo PT. Modulation of PT determines the apoptosis-inducing effect of mitochondria in a cell-free system. Moreover, inhibition of PT by BA, a specific ligand of one PT pore constituent, reduces naturally occurring apoptosis, and Bcl-2 apparently functions as an endogenous PT inhibitor. Although these findings establish mitochondrial PT as a critical event in early apoptosis, they do not resolve a number of issues concerning the cellular biology of apoptosis.

According to studies performed in *Caenorhabditis elegans*, at least two gene products, *ced-3*, which encodes a cysteine protease, and *ced-4*, whose function is unknown, are required for apoptosis to occur (62). At present, the sequence

of events that eventually link *ced-3*-like proteases and *ced-4* to mitochondria remains unknown. At present, it appears clear that both Bcl-2 (which controls PT; Figs. 7 and 8) and protease activation control two checkpoints of the apoptotic cascade (63). Tetrapeptide inhibitors of the *ced-3* homologue CPP32/Yama and of ICE fail to interfere with the induction of PT in isolated mitochondria. Moreover, they fail to inhibit the mitochondria-mediated induction of nuclear apoptosis (Table 1). When thymocyte apoptosis is induced by Fas/CD95 cross-linking, inhibition of ICE prevents both the nuclear manifestations of apoptosis and the $\Delta\Psi_m$ disruption (Marchetti, P., and G. Kroemer, unpublished results). This may indicate that at least some of the members of the family of *ced-3*-like proteases regulate events that are upstream of mitochondria. At present, however, our data cannot distinguish between two alternative possibilities. First, the PT and the protease-regulated checkpoints of the apoptotic effector phase could be placed in a serial (hierarchical) fashion. Second, both protease activation and PT could form part of parallel pathways culminating in nuclear apoptosis.

It remains largely unknown how Bcl-2 regulates PT on the molecular level. Bcl-2 does not prevent PT as such; it prevents the induction of PT by determined stimuli such as Atr, mClCCP, and *ter*-BHP, but not calcium or diamide (Figs. 7 and 8). Bcl-2 could act via direct molecular association with constituents of the PT pore, a possibility that is suggested by the localization of both Bcl-2 and PT pore constituents at inner-outer membrane contact sites (55–57). Alternatively, Bcl-2 could affect PT indirectly. Thus, it enhances oxidative phosphorylation (64) and causes mitochondrial inner membrane hyperpolarization (65), which in turn would reduce the probability of PT (24). It has previously been reported that mitochondrial membrane localization is necessary to mediate Bcl-2 suppression of apoptosis, namely when apoptosis is induced by E1B-defective adenovirus (57) and when it is triggered by IL-3 starvation of IL-3-dependent 32D cells (55). In contrast, in some other systems of apoptosis induction, a mutated Bcl-2 molecule lacking the membrane localization domain (4, 58), as well as the naturally occurring apoptosis-inhibitory Bcl-2 analogue Bcl-X Δ TM (a splice variant of Bcl-X that lacks the transmembrane domain; 66), maintain their antiapoptotic potential. However, the fact that soluble, ubiquitous Bcl-2 still maintains at least part of its antiapoptotic function does not formally exclude that it acts on the external membrane of mitochondria. The present data suggest an intimate linkage between Bcl-2 and mitochondrial regulation. In this context it may be intriguing that the *C. elegans* *bcl-2* homologue, *ced-9*, is an element of a polycistronic locus that also contains *cyt-1*, a gene that encodes a protein similar to cytochrome b560 of the mitochondrial respiratory chain complex II (67). Thus both functional and genetic evidence link Bcl-2 to mitochondrial regulation. Irrespective of the exact molecular mechanism by which Bcl-2 affects PT, the finding that Bcl-2 does inhibit PT, at least in response to certain stimuli (Figs. 7 and 8), provides an explanation for hitherto apparently contradictory reports.

Bcl-2 hyperexpression has been reported to inhibit the production and/or adverse effects of ROS (58, 68), that in turn, however, are not obligatory for apoptosis (16). In accord with these findings, Bcl-2 prevents oxidant-mediated PT (Fig. 8). Moreover, it prevents the mitochondrial ROS formation that is secondary to PT (12). Thus, Bcl-2 impedes PT as well as two dissociable consequences of PT: (a) nuclear apoptosis, and (b) mitochondrial uncoupling and superoxide anion generation.

A further issue that remains to be elucidated is the molecular mechanism by which isolated mitochondria undergoing PT cause nuclear chromatin condensation and endonuclease activation. It appears clear that this mechanism is neither cell type nor species specific, given that, for example, mouse liver mitochondria in PT can promote the apoptotic disintegration of nuclei purified from human fibroblast-like nuclei (Fig. 1). Our data indicate that mitochondria contain or are associated with (a) pre-formed soluble mediator(s) >10kD that is/are released after PT and that alone is/are sufficient to cause nuclear apoptosis (Fig. 9). In accord with published experiments performed on intact cells (16, 17), antioxidants do not neutralize this apoptosis inducer (Fig. 9). Thus, ROS that are formed by mitochondria after PT do not participate in the induction of nuclear apoptosis; this is also indicated by experiments involving ρ^0 cells that lack a functional respiratory chain (15, and Fig. 2). Moreover, it appears improbable that Ced-3-like proteases would be responsible for this apoptosis-inducing activity, given that the mammalian Ced-3 analogue CPP32 per se is not sufficient to induce nuclear apoptosis in a cell-free system (6). Thus, the molecular events linking mitochondrial PT to nuclear apoptosis await further characterization.

From the available data, it appears that $\Delta\Psi_m$ disruption, which presumably is mediated by PT, is a constant feature of early apoptosis (8–14). Indirect biochemical evidence has previously accused PT to participate in the postischemic or toxin-mediated death of myocardial cells and hepatocytes (69–72), thus again suggesting that PT is a general regulator of cell death. Indeed, the PT pore is an attractive candidate for a death switch that, once activated, marks a point of no return in PCD. At least six reasons support this concept. First, as shown here, PT is both necessary and sufficient to cause nuclear apoptosis. Second, opening of PT pores entails multiple potentially lethal alterations of mitochondrial function (loss of $\Delta\Psi_m$, uncoupling of the respiratory chain, hypergeneration of ROS, and loss of mitochondrial glu-

tathione and calcium; 12, 19–21) and thus may initiate pleiotropic death pathways. Moreover, as shown here, PT triggers a nuclear apoptosis effector pathway whose biochemical components remain elusive. Third, the PT pore functions as a sensor for multiple physiological effectors (divalent cations, ATP, ADP, NAD, $\Delta\Psi_m$, pH, thiols, and peptides), thereby integrating information on the electrophysiological, redox, and metabolic state of the cell (19, 20, 73, and Fig. 3). Thus, different death inducers can converge at this level. Fourth, given that a PT pore constituent such as the ANT is essential for energy metabolism, mutations in this apoptosis-regulatory device will be mostly lethal for the cell. In teleological terms, this would have the advantage of precluding apoptosis-inhibitory (oncogenic) mutations at this level of the apoptotic cascade. Fifth, at least one of the PT constituents, the ANT, is encoded by several members of a gene family that are expressed in a strictly tissue-specific manner (74). Thus, PT pores may be regulated in each cell type in a slightly different fashion. Sixth, PT is endowed with self-amplificatory properties in the sense that loss of matrix Ca^{2+} and glutathione, depolarization of the inner membrane, and increased oxidation of thiols, that result from PT pore opening, all increase the PT pore-gating potential (19–21, 23–29). The self-amplificatory property of PT is also underscored by the data presented in this paper. Thus, induction of PT induces $\Delta\Psi_m$ disruption (Figs. 1 and 7) and, conversely, $\Delta\Psi_m$ depolarization by mClCCP causes PT, measured as large amplitude swelling (Figs. 3 and 8). Similarly, oxidant treatment causes PT (Figs. 3 and 8), and PT will ultimately entail mitochondrial generation of ROS (12). The fact that some consequences of PT (e.g., $\Delta\Psi_m$ dissipation, ROS generation) themselves may cause PT suggests that PT may engage in a positive feedback loop that contributes to apoptotic autodestruction. Thus, PT would have to respond in an all-or-nothing fashion and, once activated, would seal the cell's fate in an irreversible fashion. Accordingly, cells exhibiting an immediate consequence of PT, that is $\Delta\Psi_m$ reduction, are irreversibly committed to cell death (10).

Apart from these theoretical considerations, the current data suggest that the PT pore occupies a central position in apoptosis regulation. It therefore becomes an attractive target for regulation by pharmacological agents, as well as by endogenous apoptosis regulators belonging to the ever-expanding Bcl-2 gene family.

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