

Bcl-2 Inhibits the Mitochondrial Release of an Apoptogenic Protease

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Summary

Bcl-2 belongs to a family of apoptosis-regulatory proteins which incorporate into the outer mitochondrial as well as nuclear membranes. The mechanism by which the proto-oncogene product Bcl-2 inhibits apoptosis is thus far elusive. We and others have shown previously that the first biochemical alteration detectable in cells undergoing apoptosis, well before nuclear changes become manifest, is a collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$), suggesting the involvement of mitochondrial products in the apoptotic cascade. Here we show that mitochondria contain a pre-formed ~50-kD protein which is released upon $\Delta\Psi_m$ disruption and which, in a cell-free in vitro system, causes isolated nuclei to undergo apoptotic changes such as chromatin condensation and internucleosomal DNA fragmentation. This apoptosis-inducing factor (AIF) is blocked by *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk), an antagonist of interleukin-1 β -converting enzyme (ICE)-like proteases that is also an efficient inhibitor of apoptosis in cells. We have tested the effect of Bcl-2 on the formation, release, and action of AIF. When preventing mitochondrial permeability transition (which accounts for the pre-apoptotic $\Delta\Psi_m$ disruption in cells), Bcl-2 hyperexpressed in the outer mitochondrial membrane also impedes the release of AIF from isolated mitochondria in vitro. In contrast, Bcl-2 does not affect the formation of AIF, which is contained in comparable quantities in control mitochondria and in mitochondria from Bcl-2-hyperexpressing cells. Furthermore, the presence of Bcl-2 in the nuclear membrane does not interfere with the action of AIF on the nucleus, nor does Bcl-2 hyperexpression protect cells against AIF. It thus appears that Bcl-2 prevents apoptosis by favoring the retention of an apoptogenic protease in mitochondria.

Before cells manifest nuclear signs of apoptosis, they disrupt the mitochondrial transmembrane potential ($\Delta\Psi_m$)¹. This pre-apoptotic $\Delta\Psi_m$ collapse occurs in nu-

merous cell types (neurons, fibroblasts, myelomonocytic cells, lymphocytes, hepatocytes), irrespective of the apoptosis inducer or the presence of mitochondrial DNA (1–5 and references cited therein). As to the mechanism of the pre-apoptotic $\Delta\Psi_m$ disruption, it appears that it is mediated by so-called “permeability transition (PT) pores”, i.e., regulated megachannels that allow for the dissipation of inner transmembrane ion gradients. Thus, a series of drugs known for their PT-inhibitory potential (6, 7) such as cyclosporin A (which acts as a transient inhibitor of PT, via mitochondrial cyclophilin) or bongkreikic acid (which acts as a stable inhibitor of PT, via specific binding to the inner membrane adenine nucleotide translocator) inhibit the pre-apoptotic $\Delta\Psi_m$ collapse (2, 4). Bongkreikic acid is also an efficient inhibitor of other manifestations of apoptosis at the levels of the nucleus (chromatin condensation and DNA fragmentation), of the cytoplasm (depletion of non-oxidized glutathione and hyperproduction of reactive oxygen

¹Abbreviations used in this paper: Ac-DEVD.CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD.CHO; acetyl-Tyr-Val-Ala-Asp-aldehyde; Ac-YVAD.cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; AIF, apoptosis-inducing factor; Atr, atractyloside; CMXRos, chloromethyl-X-rosamine; $\Delta\Psi_m$, mitochondrial transmembrane potential; mClCCP, carbonylcyanide *m*-chlorophenylhydrazone; Neo, neomycin resistance gene; MDH, malate dehydrogenase; MAO, monoamine oxidase; PI, propidium iodide; PT, permeability transition; ROS, reactive oxygen species; SDH, succinate dehydrogenase; *t*-BHP, *tert*-butylhydroperoxide; TLCK, *N*-tosyl-L-lysyl chloromethylketone; TPCK, *N*-tosyl-L-Phe-chloromethylketone; PARP, poly (ADP-ribose) polymerase; Z-D.CH2-dcb, *N*-benzyloxycarbonyl-Asp-CH₂OC(O)-2,6-dichlorobenzene; Z-FA.fmk, *N*-benzyloxycarbonyl-Phe-Ala-fluoromethylketone; Z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

S.A. Susin and N. Zamzami contributed equally to this paper.

species), and of the plasma membrane (exposure of phosphatidylserine residues in the outer membrane leaflet, increase in permeability) (4, 8). Altogether these findings suggest that mitochondrial PT may be an important common event of the effector phase of the apoptotic process. This idea is underlined by the observation that mitochondria undergoing PT acquire the capacity to induce nuclear apoptosis in an *in vitro* system (4). A further argument in favor of the implication of PT in apoptosis regulation is the finding that the apoptosis-inhibitory proto-oncogene product Bcl-2 functions as an endogenous PT inhibitor. This PT-protective Bcl-2 effect has been observed in the overall context of the intact cell (2), as well as in isolated mitochondria (4), suggesting that PT may indeed constitute the (or one of the) Bcl-2-regulated checkpoint(s) of the apoptotic cascade. It has remained obscure, however, how the putative Bcl-2/PT checkpoint could be related to another apoptosis checkpoint controlled by a cascade of proteases belonging to the expanding ICE/ CPP32/ Ced-3 family (9–11), especially in view of the fact that Bcl-2 can regulate the activation of at least some of these proteases (12).

Based on the findings of other investigators (13–17), we have recently developed a cell-free system of apoptosis. Using such a system, we observed that isolated mitochondria induced to undergo PT *in vitro* release a heat-labile >10-kD factor capable of inducing isolated nuclei to rapidly (≤ 15 min) manifest apoptotic changes such as chromatin condensation and oligonucleosomal DNA fragmentation (4). This finding suggested a direct molecular link between pre-apoptotic $\Delta\Psi_m$ disruption and nuclear apoptosis. In the present paper, we report the biochemical characterization of the apoptogenic factor released by mitochondria. Intriguingly, this factor possesses a protease activity which is neutralized by a degenerate tripeptide inhibitor of ICE-like proteases, suggesting a direct relationship between PT and protease activation during the effector phase of apoptosis. In addition, we characterized the effect of Bcl-2 on the production, release, and action of this mitochondrial apoptosis-inducing factor (AIF). Our data indicate that Bcl-2 solely interferes with the PT-dependent release of AIF, yet does not neutralize its lethal action. These data may provide major clues for the understanding of apoptosis regulation.

Materials and Methods

Organs and Cell Lines. Livers were obtained from female Balb/c mice (6–8 wk of age) subjected to perfusion of the portal vein with isotonic PBS (pH 7.2; 5 ml at room temperature). 2B4.11 T cell hybridoma cell lines stably transfected with an SFFV.neo vector containing the human bcl-2 gene or the neomycin (Neo) resistance gene only were kindly provided by Jonathan Ashwell (National Institutes of Health, Bethesda, MD). In this cell line, Bcl-2 overexpression confers resistance to most but not all apoptosis-inducing stimuli (18). These cell lines, as well as human fibroblast-like HeLa cells, were cultured in RPMI1640 medium supplemented with l-glutamine, antibiotics, and 10% de complemented FCS.

Induction of Apoptosis in Cells. T cell hybridoma cells were cul-

tured during 8 h in the presence of the indicated concentration of *tert*-butylhydroperoxide (*t*-BHP), carbonylcyanide *m*-chlorophenylhydrazone (mCICCP), C₂-ceramide (Sigma, Chem Co., St. Louis, MO), *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk; Enzyme Systems, Dublin, CA), and/or the membrane-permeant specific inhibitor of interleukin-1 β -converting enzyme (ICE) Ac-YVAD.cmk (Bachem, Basel, Switzerland). The frequency of subdiploid cells was determined by propidium iodide staining of ethanol-fixed cells. In some experiments, Bcl-2-or vector-transfected cells (1×10^6) were permeabilized with saponin (5 mg/ml) in the presence of CFS buffer (see below) or purified AIF (1 μ g/300 μ l), then washed twice (1,000 g, 10 min), and cultured for 60 min in CFS buffer. Then, DNA fragmentation was assessed using terminal deoxynucleotidyltransferase to incorporate Fluorescein-12-dUTP into permeabilized cells, following the manufacturer's protocol (TUNEL method; Boehringer Mannheim, Mannheim, Germany).

Purification of Organelles. Mitochondria were purified on a Percoll® (Pharmacia, Uppsala, Sweden) gradient (19) and were stored on ice in B buffer (400 mM mannitol; 10 mM KH₂PO₄, 5 mg/ml BSA, 50 mM TRIS-HCl, pH 7.2) for up to 4 h. Mitochondria were washed and resuspended in cell-free system (CFS) buffer (220 mM mannitol; 68 mM sucrose, 2 mM NaCl, 2.5 mM PO₄H₂K, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM phenylmethyl sulfonylfluoride [PMSF], 1 mM dithiothreitol, 10 mM Hepes-NaOH, pH 7.4) before manipulation (4). Submitochondrial fractions were obtained as described (20). Osmotic shock of mitochondria leading to the disruption of the outer but not of the inner membrane or digitonin lysis (1.2%) of the outer mitochondrial membrane for the obtention of intermembrane proteins was performed according to standard methods (20). The four mitochondrial compartments (inner + outer membrane, intermembrane, matrix) were identified by the specific activities of the marker enzymes monoamine oxidase (MAO) for the outer membrane, succinate dehydrogenase (SDH) for the inner membrane, and malate dehydrogenase (MDH) for the matrix (21). Enzyme activities were determined as described (21) and expressed as specific activities (nmol of transformed substrate \times mg⁻¹ total protein \times min⁻¹). Freeze-thawing of intact mitochondria was performed in two cycles (liquid nitrogen followed by 37°C during 5 min). Ultrasonication was carried out using a Branson sonicator (model 130; Danbury, CT; 20 kHz, 400 W, 5 min, 4°C). In one experiment, the AIF-containing intermembrane fraction was treated by proteinase K digestion (100 μ g/ml, 10 min at 37°C; Boehringer Mannheim, Mannheim, Germany), followed by proteinase K inactivation by addition of 1 mM PMSF (22). Nuclei from the indicated cell type were purified on a sucrose gradient and conserved in 50% glycerol (Sigma) in HNB buffer, at 20°C for a maximum of 15 d, as described (13, 23). The expression of Bcl-2 on the surface of isolated mitochondria or nuclei was determined using an FITC-labeled hBcl-2-specific antibody (1 μ g/ml, 30 min at 4°C, clone 124; DAKO, Glostrup, Denmark).

Determination of Apoptogenic Effects on Isolated Nuclei. For the standard assessment of chromatin condensation, nuclei from HeLa cells were purified on a sucrose gradient as above, washed 2 \times (1,000 g; 10 min, 4°C) and resuspended in CFS buffer. In standard conditions, nuclei (10³ nuclei/ μ l) were cultured in the presence of mitochondrial preparations for 90 min at 37°C. Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 μ M; Molecular Probes, Leiden, The Netherlands) and examined by fluorescence microscopy (13, 14). A minimum of 200 events were scored and nuclei presenting chromatin conden-

sation and/or a translucent aspect were interpreted as being apoptotic (4). Alternatively, nuclei were stained with propidium iodide (PI; 10 $\mu\text{g}/\text{ml}$; Sigma) and the lipophilic dye 5-methyl-bodipy-3-dodecanoic acid (100 nM; Molecular Probes), followed by cytofluorometric analysis in a EPICS Profile II Analyzer (Coulter, Hialeh, FL). Only membrane-surrounded (5-methyl-bodipy-3-dodecanoic acid-labeled) particles were gated on. A good correlation between the frequency of nuclei exhibiting chromatin condensation with DAPI and hypoploidy with PI was obtained (S.A. Susin and G. Kroemer, manuscript in preparation). In one series of experiments, nuclei from Neo- or Bcl-2-transfected T cell hybridoma cells were subjected to transmission electron microscopy, as described (8). Furthermore, in one experiment, nuclei were pretreated with Z-VAD.fmk (1 mM, 60 min, 37°C in CFS buffer), washed 3 \times (1000 g; 10 min), and exposed to mitochondrial factors.

Purification of the Mitochondrial Apoptosis-inducing Factor (AIF). Purified hepatocyte mitochondria were treated with atractyloside (5 mM; Atr; Sigma) to induce PT and liberation of AIF (4). Supernatants (150,000 g; 1 h; 4°C) from these mitochondria were concentrated on Centricon 10 membranes (≥ 10 kD; Amicon Inc., Beverly, MA), which retains the entire AIF activity (4), and then subjected to a Pharmacia MonoQ (HR5/5) FLPC column pre-equilibrated with protein-free CFS buffer. Elution was performed on a linear gradient from 0 to 250 mM NaCl at 0.5 ml/min over 30 min, followed by elution at 1 M from min 33. After dialysis (10 kD membrane) in CFS buffer, all fractions were subjected to the assessment of pro-apoptotic activity. The active fraction (eluting at 110 mM NaCl) was dialyzed against protein-free CFS buffer (4°C, 4 h, 5,000 \times excess of CFS buffer) and subjected to re-injection into the Pharmacia MonoQ FLPC column (5 μg ; elution as described above) or a reverse phase C-18 column coupled to a Hewlett Packard 1090 HPLC apparatus (eluted on a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile at 1 ml/min over 30 min). The SDS-PAGE (12%; performed in reducing conditions: 1 mM dithiothreitol) of AIF purified by anion exchange chromatography (concentrated on a Centricon 10 membrane; 2 μg) was subjected to Silver staining (BioRad, Hercules, CA). Alternatively, AIF-containing supernatants or AIF purified on the MonoQ column were fractionated on a Pharmacia Superdex75 column equilibrated with CFS buffer. All fractionation steps had to be carried out at 4°C to avoid loss of biological AIF activity. To determine the inhibitory profile of AIF, different protease inhibitors were added to purified AIF or AIF-containing preparations: Z-VAD.fmk; *N*-benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA.fmk; Enzyme Systems), *N*-tosyl-L-Lys-chloromethylketone (TLCK; Sigma), *N*-tosyl-L-Phe-chloromethylketone (TPCK), E64, leupeptin, *p*-chloromercuriphenylsulfonic acid, *N*-phenylmaleimide (Sigma), calpain inhibitors I and II (Boehringer), or modified tetrapeptides and *N*-benzyloxycarbonyl-Asp-CH₂OC(O)-2-6,-dichlorobenzene (Z-D.CH₂-dcb; Bachem).

Quantitation of Mitochondrial Permeability Transition (PT). For the induction of PT, mitochondria from different cell lines were incubated with the indicated doses of Atr, *t*-BHP or mClCCP (4). The $\Delta\Psi_m$ was measured using the $\Delta\Psi_m$ -sensitive cationic lipophilic dye chloromethyl-X-rosamine (CMXRos; 10 nM; 15 min at 37°C; Molecular Probes) (5), after having added the indicated PT inducer (15 min, 37°C). Mitochondria were analyzed in an Elite cytofluorometer (Coulter). All $\Delta\Psi_m$ determinations were performed at least three times in each experiment.

Determinations of Protease and DNase Activity. AIF-mediated cleavage of nuclear substrates is indicated by the comparative analysis of

Coomassie-stained SDS-PAGE of HeLa nuclei (5 $\times 10^6$ /lane) cultured in the presence or absence of 100 ng/ml purified AIF (90 min, 37°C). The degree of poly(ADP-ribose) polymerase (PARP) and lamin cleavage was determined by immunoblots, using the monoclonal antibodies C2-10 (purchased from Guy Poirier, Montreal University, Canada) and E6 (kind gift from Dr. E. Nigg, Lausanne University, Lausanne, Switzerland), respectively (14, 24, 25). Positive controls were human T lymphoma cells exposed to anti-Fas antibody (500 ng/ml; 4 h; Immunotech, Marseille, France). Endonuclease activity was determined on supercoiled pUC DNA (500 ng in CFS buffer; 120 min; 37°C), followed by ethidium bromide agarose gel (1%) electrophoresis.

Results

Localization and Purification of AIF. Supernatants from mitochondria that have undergone PT but not those from control mitochondria induce signs of apoptosis such as nuclear chromatin condensation in a cell free system (4). Thus, mitochondria treated with the PT inducers atractyloside (Atr, a specific ligand of the adenine nucleotide translocator), mClCCP (a protonophore) and *t*-BHP (a prooxidant) release an apoptogenic activity into the supernatant that we have baptized "apoptosis-inducing factor" (AIF) (Fig. 1). In addition to these PT-inducing treatments, destruction of mitochondrial membranes via sonication, osmotic shock, or digitonin treatment (which specifically lyses the outer but not the inner membrane, yielding the intermembrane fraction of proteins) (20) also releases AIF, indicating that AIF is pre-formed. Whereas, the fraction of soluble intermembrane products does contain AIF activity, neither purified mitochondrial membranes nor the soluble products of the mitochondrial matrix are apoptogenic (Fig. 1, Table 1). Thus, AIF is not found in submitochondrial fractions that contain high specific activities of marker enzymes for the outer membrane (MAO), inner membrane (SDH), and matrix (MDH) (Table 1). Proteinase K treatment of intermembrane proteins destroys AIF activity, indicating that AIF is a protein (Fig. 1). Ion exchange chromatography yields only one AIF activity which elutes at 110 mM NaCl (Fig. 2 A). The AIF-containing fraction purified on an anion exchange column contains only one major protein as determined by four different methods: reinjection into the same anion exchange FPLC column (Fig. 2 B), analysis on a reverse phase HPLC column (which separates proteins based on their hydrophobicity; Fig. 2 C), separation in a molecular sieve FPLC column (Fig. 2 D) and SDS-PAGE revealed by the sensitive silver staining technique (Fig. 2 E). Thus, anion exchange chromatography (Fig. 2 A), molecular sieve chromatography (Fig. 2 D), and SDS-PAGE (Fig. 2 E) identify hepatocyte AIF as a single ~ 50 -kD protein with an estimated isoelectric point of 5.5. AIF purified on an anion exchange column (Fig. 2 A) is homogenous with regard to charge (Fig. 2 B), hydrophobicity (Fig. 2 C), and apparent molecular weight in both denaturing and non-denaturing conditions (Fig. 2, D and E). AIF is low-abundant (<0.1% of mitochondrial proteins) and labile at room temperature, requiring purification at 4°C. AIF activity is

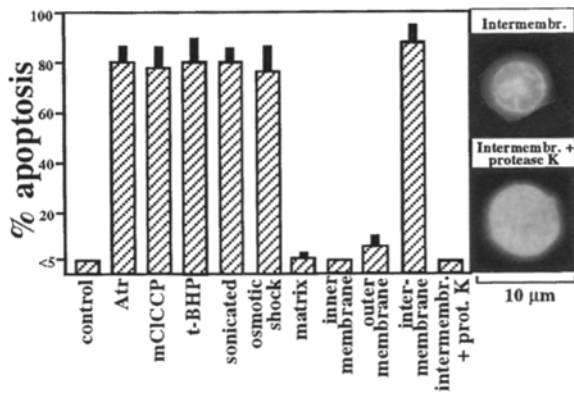


Figure 1. Submitochondrial localization of mitochondrial apoptosis-inducing factor (AIF). Isolated HeLa nuclei were cultured in the presence of various preparations of mouse hepatocyte mitochondria: ultracentrifuged supernatants of mitochondria (10 mg protein/ml) treated with Atr (5 mM), mCICCP (100 μ M), *t*-BHP (50 μ M), osmotic shock, or ultrasonication; soluble products (200 μ g protein/ml) of the mitochondrial matrix, purified inner and outer mitochondrial membranes, or soluble factors of the intermembrane space. After 90 min of culture at 37°C the frequency of nuclei exhibiting chromatin condensation was assessed by labeling with DAPI. Proteinase K digestion of intermembrane proteins was followed by addition of 1 mM PMSF for its inactivation, as described in Materials and Methods. Representative DAPI-stained HeLa nuclei (>70 % of the phenotype) cultured in the presence of untreated or proteinase K-inactivated intermembrane preparation are shown.

detected in mitochondria from several cell types (liver, heart, brain, myelomonocytic cells, lymphoid cells) and species (mouse, human), suggesting that it is an ubiquitous protein. Moreover, the physicochemical characteristics of this protein appear approximately the same for hepatocyte and lymphocyte AIF (not shown). Purified AIF suffices to induce the whole pattern of apoptotic nuclear alterations in vitro: eccentric DNA condensation resembling that observed in apoptotic cells, oligonucleosomal DNA fragmentation, and hypoploidy (Figs. 3 and 6 C; see below).

In synthesis, it appears that AIF is one pre-formed, soluble mitochondrial intermembrane protein.

AIF Possesses a Proteolytic Activity That Is Inhibited by Z-VAD.fmk. To gain information on the mode of action of AIF, we determined the inhibitory profile of this factor. The chromatin condensation-inducing activity of AIF is inhibited by the thiol reagents *p*-chloromercuriphenylsulfonic acid (ID_{50} \sim 250 μ M) and *N*-phenylmaleimide (ID_{50} \sim 50 μ M) but not by specific inhibitors of different calcium, serine, or cysteine proteases including specific inhibitors of ICE (Ac-YVAD.cmk, Ac-YVAD.CHO: no inhibition at doses as high as 1 mM) and CPP32/Yama (Ac-DEVD.CHO) (reference 4 and Fig. 3 A). The only selective protease inhibitor which blocks AIF activity is Z-VAD.fmk (Fig. 3 A; ID_{50} \sim 30 μ M), an inhibitor of ICE-like proteases

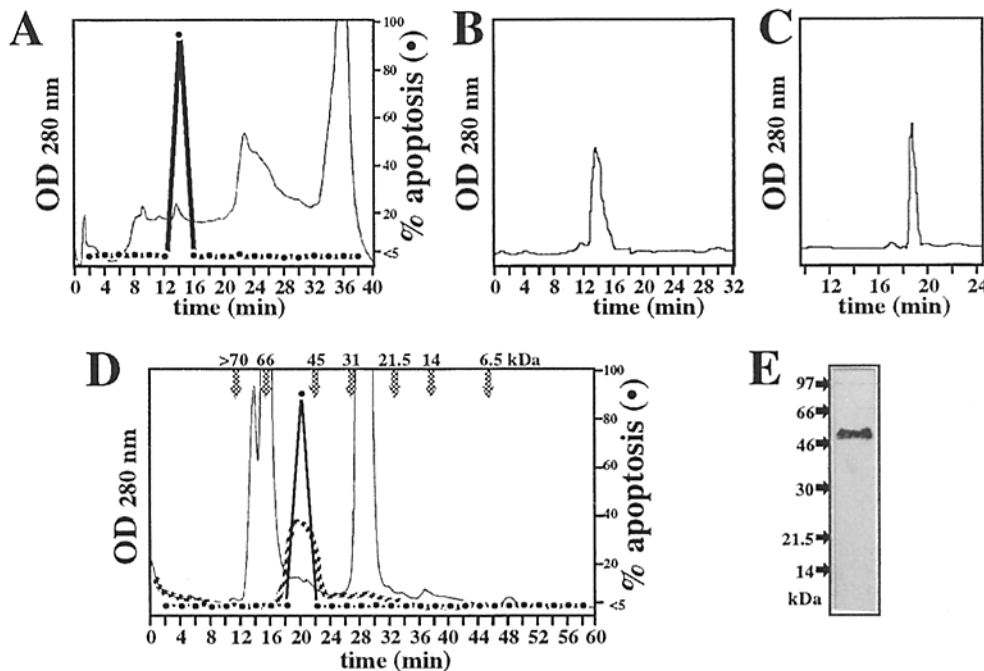


Figure 2. Biochemical characterization of AIF. (A) Anion exchange purification of AIF. Concentrated supernatants from Atr-treated hepatocyte mitochondria were subjected to a MonoQ FLPC column pre-equilibrated with CFS buffer. Elution was performed on a linear gradient from 0 to 250 mM NaCl at 0.5 ml/min over 30 min while monitoring the OD280 (full line). After dialysis (10 kD membrane) in an excess of CFS buffer, all fractions were subjected to the assessment of pro-apoptotic activity (circles). Only one fraction eluting at 110 mM NaCl was biologically active. (B) Re-injection of the AIF-containing fraction eluting at 110 mM into an anion exchange column. AIF (5 μ g protein) purified as in A and dialyzed in excess CFS buffer was injected into a MonoQ column in the same conditions of elution as in A, and the OD280 was monitored. The background

OD280 obtained with CFS buffer only was subtracted. (C) Analysis of AIF (5 μ g, purified on an anion exchange column as in A) in a reverse phase HPLC column, followed by monitoring of the OD280 (background values subtracted). (D) Molecular sieve FPLC purification of AIF. AIF-containing supernatants (OD280: full line) were fractionated on a Superdex75 column equilibrated with CFS buffer, followed by the biological assay (circles). Proteins with a known molecular weight were employed to calibrate the FPLC column (arrows). Equivalence calculations indicate that \geq 80% of AIF activity are recovered from the columns. In addition, AIF (5 μ g) purified on the anion exchange column as in A was analyzed on the Superdex 75 column while monitoring the OD280 (dotted line). (E) SDS-PAGE of AIF (2 μ g; purified as in A) in reducing conditions. Proteins were detected using a commercial Silver staining kit, as described in Materials and Methods.

Table 1. Distribution of AIF in Mitochondrial Compartments

	Marker enzymes			AIF Activity
	MAO	SDH	MDH	
		<i>mmol/mg/min ± SD</i>		<i>% apoptosis</i>
Mitochondria (freeze-thawed)	52 ± 9	517 ± 149	2812 ± 265	67 ± 12
Outer membrane	503 ± 46	555 ± 186	1028 ± 364	14 ± 6
Intermembrane space	2 ± 1	10 ± 6	831 ± 220	85 ± 7
Inner membrane	2 ± 2	2411 ± 268	1416 ± 184	<5
Matrix	1 ± 1	<5	4054 ± 880	8 ± 3

Submitochondrial fractions were purified as described in Materials and Methods. Marker enzymes were measured using colorimetric approaches on synthetic substrates. AIF activity was determined for each fraction (200 µg protein/ml) as in Fig. 1. Results are mean values ± SD of four independent experiments.

(26–28). By virtue of its tripeptidic structure, Z-VAD.fmk is thought to be a less specific protease inhibitor than conventional tetrapeptidic inhibitors of ICE or CPP32/Yama (26–28). Z-VAD.fmk does not neutralize AIF by virtue of its *N*-benzoylcarbonyl (Z) or fluoromethylketone (fmk)

groups, because the control molecule Z-FA.fmk (which only differs from Z-VAD.fmk in its amino acid sequence: FA instead of VAD) does not inhibit AIF activity at doses as high as 1 mM (Fig. 3 A and data not shown). Altogether these data suggest that AIF possesses a cysteine-dependent

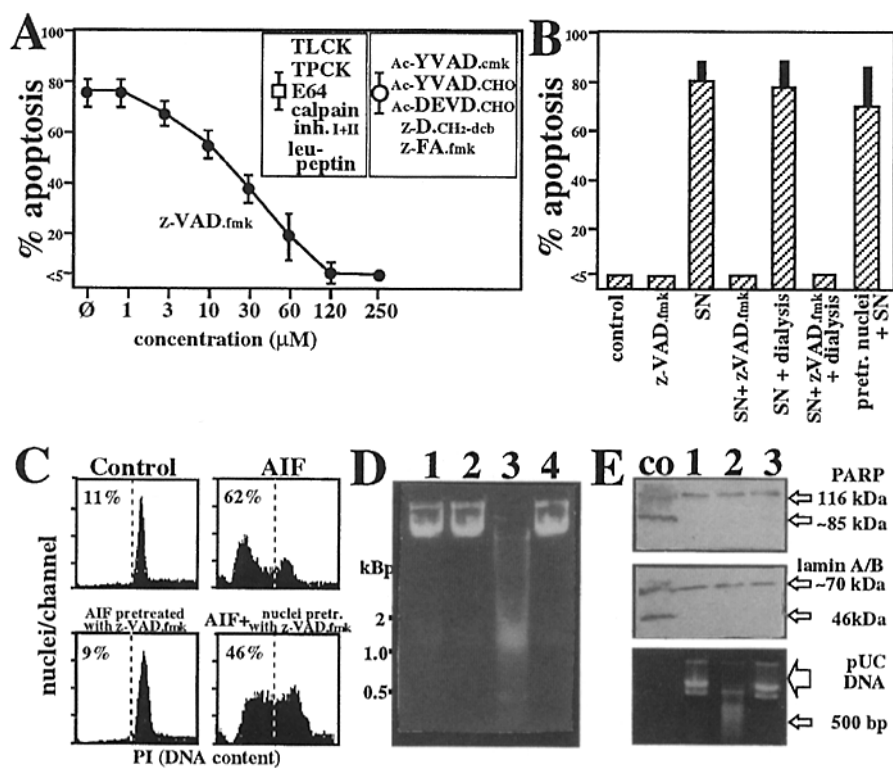


Figure 3. Inhibitory profile of AIF. (A) Screening of various protease inhibitors. Purified AIF (50 ng/ml) was tested for apoptosis-inducing activity on HeLa nuclei as in Fig. 1, in the presence of a number of different protease inhibitors. The range of negative data are shown for different inhibitors used at concentrations of either 50 µM (TLCK, TPCK, E64, calpain inhibitors I and II, leupeptin) or 250 µM (Ac-YVAD.cmk, Ac-YVAD.CHO, Ac-DEVD.CHO, Z-D. CH₂-dcb, Z-FA.fmk). (B) Inhibition of the AIF activity contained in supernatants from Atr-treated mitochondria. Nuclei were treated at the same time with such supernatants and/or 100 µM Z-VAD.fmk. Alternatively, the supernatant was pretreated with Z-VAD.fmk (100 × µM; 30 min, 37°C), followed by dialysis (4°C, 4 h, 5,000× excess of CFS buffer) for removal of Z-VAD.fmk and testing for apoptotic activity on isolated HeLa nuclei. Furthermore, nuclei were pretreated with Z-VAD.fmk (1 mM, 60 min, 37°C in CFS buffer), washed 3×, and exposed to the untreated AIF-containing supernatant. (C) Z-VAD.fmk-mediated inhibition of AIF-induced chromatinolysis. Nuclei were treated with CFS buffer only (control), purified AIF (100 ng/ml), or AIF pretreated with Z-VAD.fmk and dialysis. Alternatively, nuclei were first treated with Z-VAD.fmk, then washed and

cultured with AIF. Nuclei were stained with PI, as detailed in Materials and Methods, followed by cytofluorometric analysis. Percentages refer to the subdiploid population. (D) Z-VAD.fmk-mediated inhibition of AIF-induced DNA fragmentation. Isolated nuclei (1 × 10⁶) were cultured during 90 min in the presence of purified AIF (100 ng/ml, lanes 3 and 4) and/or Z-VAD.fmk (100 µM, lanes 2 and 4) and analyzed by ethidium bromide agarose gel electrophoresis. (E) Effect of AIF on protease substrates and purified DNA. HeLa nuclei were exposed to CFS buffer only (1), the supernatant of digitonin-permeabilized mitochondria (2), or 100 ng/ml purified AIF (3). The degree of PARP and lamin cleavage was determined by immunoblots. Positive controls (co) are human lymphoma cells exposed to anti-Fas antibody. The same three preparations were tested for endonuclease activity on pUC DNA, followed by ethidium bromide agarose gel electrophoresis.

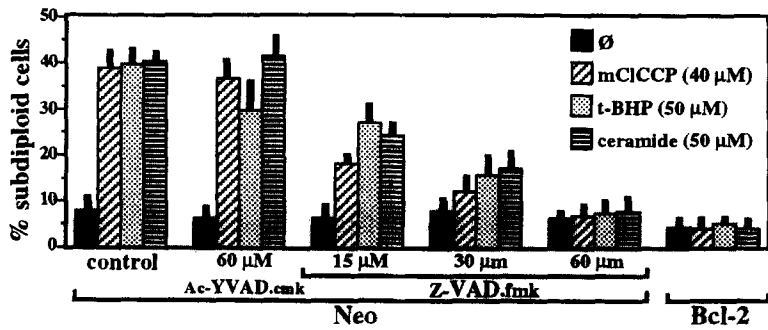


Figure 4. Effect of Z-VAD.fmk and transfection-enforced Bcl-2 hyperexpression on apoptosis induction in cells. T cell hybridoma cell lines stably transfected with vectors containing the human *bcl-2* gene or the neomycin (Neo) resistance gene only were cultured during 8 h in the presence of the indicated apoptosis inducer and/or protease inhibitor. Apoptosis (subdiploidy) was quantified by propidium iodide staining of ethanol-fixed cells.

catalytic activity not identical with but related to ICE and CPP32.

Z-VAD.fmk prevents the chromatin condensation induced by purified AIF as well as by supernatants from Atr-treated mitochondria (Fig. 3, B and C), in accord with the observation that AIF seems the only pro-apoptotic mitochondrial factor (Fig. 2, A and C; see above). Z-VAD.fmk is an irreversible rather than competitive protease inhibitor (26–28). Accordingly, pre-incubation of AIF with Z-VAD.fmk causes a durable AIF inactivation that persists after removal of Z-VAD.fmk (Fig. 3, B and C). In contrast, pre-incubation of nuclei with Z-VAD.fmk has no significant protective effect against AIF (Fig. 3, B and C), thus confirming that Z-VAD.fmk acts on AIF rather than on the nuclear target(s) of AIF. Z-VAD.fmk prevents all manifestations of apoptosis induced by AIF: chromatin condensation (Fig. 3, A and B), DNA loss from nuclei (Fig. 3 C) and oligonucleosomal DNA fragmentation (Fig. 3 D).

Mitochondria contain an endonuclease which has also been found in nuclei from thymocytes (29). Accordingly, a DNase activity is found in the supernatant of digitonin-treated mitochondria (Fig. 3 E). However, purified AIF has no intrinsic DNase activity (Fig. 3 E), indicating that it probably induces DNA fragmentation via activating pre-existing nuclear DNases. Although AIF has a proteolytic activity on unidentified nuclear substrates of approximately 28, 42, and 90 kD (not shown), it fails to cleave PARP or lamin in isolated nuclei (Fig. 3 E). Thus, the nuclear substrate(s) of AIF must be different from those cleaved by prominent members of the ICE/ CPP32 family of proteases (11, 25, 30).

If AIF was a rate-limiting factor of cell death, its inhibition should retard or inhibit nuclear apoptosis in cells. According to the literature, the AIF inhibitor Z-VAD.fmk prevents apoptosis of mammalian and insect cells responding to a variety of different lethal stimuli (26–28). In addition, as shown in Fig. 4, Z-VAD.fmk (but not its structural analogue Ac-YVAD.cmk, a specific inhibitor of ICE) inhibits apoptosis induced by ceramide, the pro-oxidant *t*-BHP, or the protonophore mClCCP, which acts directly on the inner mitochondrial membrane (Fig. 5). This effect is obtained at similar doses as those required to inactivate purified AIF (~60 μM) in vitro (Fig. 3 A), underlining the probable role of AIF in apoptosis.

In synthesis, it appears that mitochondria undergoing PT

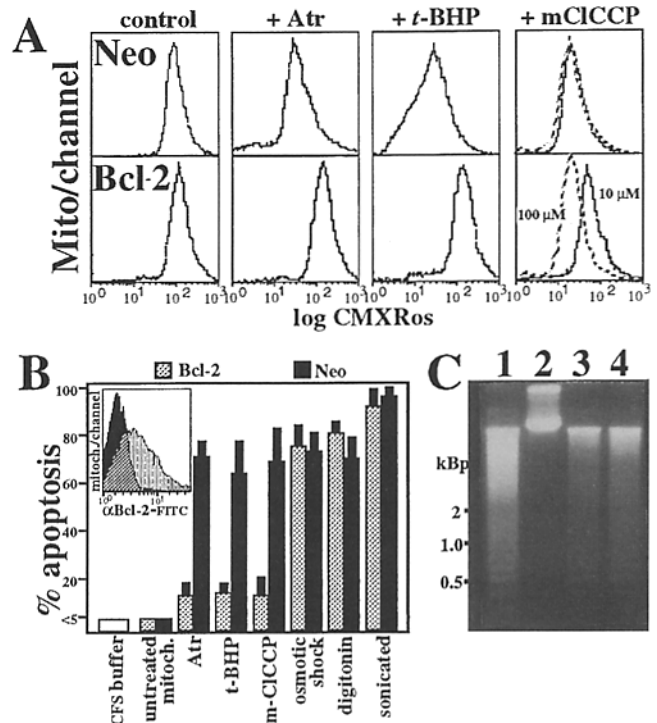


Figure 5. Bcl-2-mediated inhibition of mitochondrial permeability transition (PT)-dependent AIF release. (A) Effect of Bcl-2 on the $\Delta\Psi_m$ of isolated mitochondria exposed to different PT inducers in vitro. Mitochondria were purified from neo- or bcl-2-transfected cells, cultured during 15 min in the presence of Atr (5 mM), *t*-BHP (50 μM), or mClCCP (10 μM, continuous line; 100 μM, dashed line) and then labeled with the $\Delta\Psi_m$ -sensitive dye CMXRos. Representative cytofluorometry profiles of 2×10^4 mitochondria are shown. Note that low mClCCP concentrations reveal a difference between mitochondria from neo- and bcl-2-transfected cells but that this difference is overcome by a high mClCCP dose. Similar results were obtained when, instead of CMXRos, DiOC₆ (3) was used as a $\Delta\Psi_m$ -sensitive fluorochrome (not shown). (B) Bcl-2-mediated inhibition of AIF release from isolated mitochondria. Mitochondria from neo- or bcl-2-transfected cells were purified (6), diluted to a concentration of 10 mg/ml protein in CFS buffer, and treated during 5 min at 37°C with Atr (5 mM), *t*-BHP (50 μM), or mClCCP (50 μM). Alternatively, mitochondria from neo- or bcl-2-transfected cells were exposed to osmotic shock, ultrasonication, or the detergent digitonin, followed by centrifugation. Supernatants were tested for apoptosis induction in isolated HeLa nuclei, as in Fig. 1. The inset shows cytofluorometric profiles of purified mitochondria stained with an FITC-labeled hBcl-2-specific antibody. (C) DNA-degrading activity of supernatants from control and Bcl-2 mitochondria. Supernatants were generated from control mitochondria (1, 3) or Bcl-2-hyperexpressing mitochondria (2, 4) exposed to Atr (1, 2) or osmotic shock (3, 4) as in B and were tested for induction of DNA degradation on isolated HeLa cells as in Fig. 3 D.

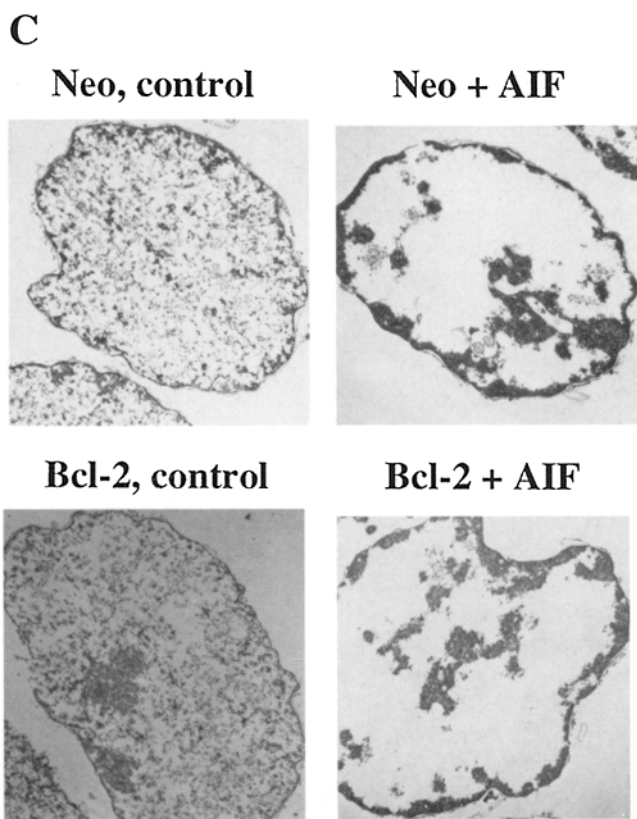
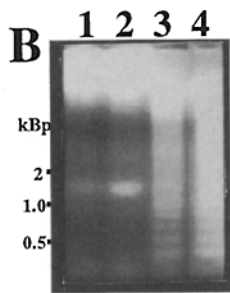
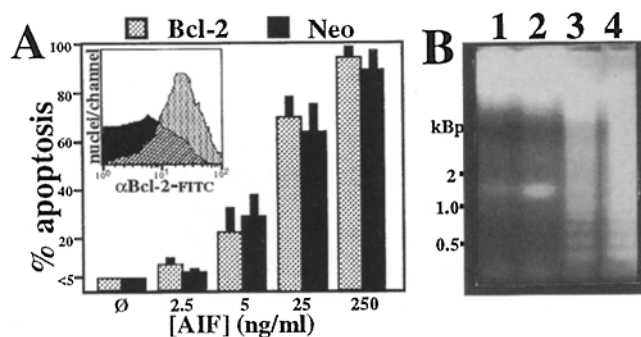


Figure 6. Effect of Bcl-2 on AIF action in vitro. (A) Comparison of AIF effects on isolated nuclei from bcl-2-transfected and control cells cultured with different concentrations of purified AIF as in Fig. 1. The insert shows cytofluorometric profiles of nuclei stained with an anti-hBcl-2-FITC conjugate as in Fig. 5 B. (B) Comparison of DNA degradation induced in nuclei from control (1, 3) or bcl-2-transfected cells (2, 4) cultured in the absence (1, 2) or presence (3, 4) of purified AIF (250 ng/ml). (C) Comparison of the ultrastructure of nuclei from Neo- or bcl-2-transfected cells cultured in the presence or absence of AIF (250 ng/ml; 90 min). Typical cells representing the dominant phenotype ($\geq 80\%$) of cultures are shown.

release a Z-VAD.fmk-inhibitable protease which is sufficient and probably necessary to cause nuclear apoptosis.

Bcl-2 Prevents the Permeability Transition-dependent Release of AIF from Mitochondria. Bcl-2 belongs to a family of apoptosis-regulatory proteins which incorporate into the outer mitochondrial membrane (31–34). Since the antiapoptotic mode of action of Bcl-2 is elusive, we addressed the question as to whether Bcl-2 inhibits apoptosis via interfer-

ing with the formation or the release of AIF. Transfection-enforced hyperexpression of Bcl-2 prevents the induction of apoptosis by *t*-BHP and the mitochondrion-targeted agent mClCCCP (reference 4 and Fig. 4). In addition, isolated mitochondria from bcl-2 transfected cells (which hyperexpress Bcl-2, see insert of Fig. 5 B) fail to disrupt their $\Delta\Psi_m$ in response to Atr, the pro-oxidant *t*-BHP, and a low dose (10 μM) of the protonophore mClCCCP (Fig. 5 A), thus confirming our previous observations according to which Bcl-2 prevents the PT-dependent colloid osmotic swelling of mitochondria in vitro (4). Simultaneously, Bcl-2 overexpressing mitochondria fail to release AIF activity in response to these PT inducers (Fig. 5, B and C), underscoring the absolute correlation between PT and AIF release that we have previously reported (4). Thus, AIF is present in the supernatants of control mitochondria treated with Atr, *t*-BHP, or mClCCCP, yet is absent or greatly reduced in the supernatant of Bcl-2 hyperexpressing mitochondria treated with these reagents. In contrast, lysis of control and Bcl-2-overexpressing mitochondria with detergents or osmotic shock releases equal amounts of AIF activity (Fig. 5, B and C).

Thus, Bcl-2 inhibits the mitochondrial AIF release in response to determined stimuli but not the formation of AIF.

Failure of Bcl-2 to Interfere with AIF Action on Nuclei and Cells. The Bcl-2 p26 protein possesses a transmembrane domain allowing for its incorporation into different intracellular membranes, including the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope (33, 34). We therefore addressed the possibility that Bcl-2 might antagonize the action of AIF. In a first series of experiments, nuclei were purified from control (Neo) and Bcl-2 transfected cells and cultured with purified AIF. Bcl-2 expressing nuclei (Fig. 6 A, inset) undergo apoptotic changes in response to AIF, exactly as this is the case for control nuclei (Fig. 6, A and B). Electron microscopic analysis confirmed that Bcl-2 hyperexpression does not affect the action of AIF. Both control and Bcl-2-overexpressing nuclei manifest the same pattern of chromatin

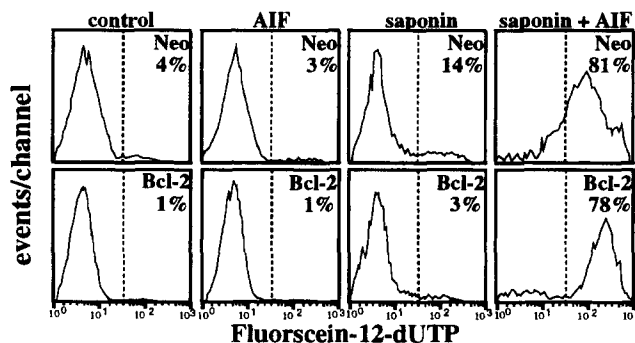


Figure 7. Effect of Bcl-2 on AIF action in cells. Failure of Bcl-2 to induce AIF resistance in cells. Bcl-2- or vector-transfected cells were permeabilized with saponin in the presence or absence of purified AIF (1 $\mu\text{g}/300 \mu\text{l}$), washed, and cultured for 60 min in CFS buffer. DNA fragmentation was assessed by the TUNEL method. Numbers indicate the percentage of cells with strong DNA fragmentation.

condensation (Fig. 6 C). Thus, nuclear expression of Bcl-2 does not affect the action of AIF.

Since Bcl-2 exists also in localizations outside of the nuclear and mitochondrial membrane (33), we decided to evaluate the effect of Bcl-2 overexpression on cells exposed to AIF. As shown above, AIF is a hydrophilic protein and thus does not permeate the plasma membrane. Accordingly, AIF only induces apoptotic changes in permeabilized cells (Fig. 7). Introduction of AIF into saponin-treated cells causes the same extent of DNA fragmentation in control and in Bcl-2-hyperexpressing cells (Fig. 7). Thus, Bcl-2 only acts upstream, at the level of AIF release, and has no detectable effect on AIF action, both in cells and in isolated nuclei.

Discussion

In this paper, we provide several new elements allowing for the construction of a scenario recreating the apoptotic effector phase and its regulation by the proto-oncogene *bcl-2*.

AIF, a Mitochondrial Apoptogenic Protease. Mitochondria undergoing PT release a protein factor, AIF, which appears to be a pre-formed, ubiquitous factor associated with the intermembrane space (Fig. 1). AIF suffices to induce typical manifestations of nuclear apoptosis in a cell-free, cytosol-free system: chromatin condensation, chromatinolysis, hypoploidy, and oligonucleosomal ladder-type DNA fragmentation (Figs 1, 3, and 6 C). AIF lacks an intrinsic DNase activity, yet seems capable of activating pre-formed nuclear DNases. Intriguingly enough, all in vitro activities of AIF are blocked by Z-VAD.fmk (Fig. 3), which is also an efficient inhibitor of apoptosis in mammalian and insect cells (26–28 and Fig. 4). Although this is not a formal proof, this finding underlines the probable importance of AIF as a rate-limiting factor of the apoptotic process in vivo. Z-VAD.fmk has been designed for the inhibition of ICE-like proteases. However, AIF is not among the known members of the family of ICE/ CPP32/ Ced3-like proteases. Its molecular mass (~50 kD), subcellular localization, and proteolytic spectrum (notably the absence of PARP cleavage) are not compatible with those of known members of the ICE/ CPP32/ Ced3 family (11). In addition, preliminary NH₂-terminal sequence data suggest that at least mouse hepatocyte AIF is encoded by a novel gene. Thus, it would be premature to consider AIF as an ICE-like protease, and the further characterization of AIF will require its cloning and genetic manipulation.

Is AIF the Principal Apoptogenic Factor? Our data suggesting a major role of AIF in the apoptotic process are compatible with previous reports that mitochondria are necessary to mediate apoptosis either in mammalian or in *Xenopus laevis* cells (15, 16) and that some known members of the ICE family such as ICE itself or CPP32 do not suffice to cause apoptosis in a cell-free system (14, 17, 30, 35). Moreover, our data are compatible with reported cell-free systems of apoptosis with regard to the particular ion requirements of AIF action, which functions in the presence of calcium chelators and at a neutral pH (13–17). In contrast,

our results appear to be in conflict with the observation that mitochondria can be substituted for by ceramide to induce nuclear apoptosis in vitro (16). However, careful analysis reveals a major functional difference between AIF and ceramide. First, the apoptosis-inducing effect of ceramide on cells is antagonized by Bcl-2 (2), which is not the case for AIF (Fig. 7). Second, maximal doses of C2 ceramide require a minimum of 3 h to induce nuclear apoptosis in vitro (16), whereas high doses of AIF act within ≤ 15 min (4). Thus, AIF rather than ceramide would be a rate-limiting apoptogenic factor. Nagata and co-workers have reported the existence of a cytosolic apoptogenic factor that is produced upon Fas-cross-linking (17, 35). The method of isolation to obtain this factor, repeated freezing and thawing, would destroy the activity of AIF. Interestingly, these authors have found that cytosolic extracts treated with recombinant CPP32 become apoptogenic (35). As a consequence, it cannot be excluded that, at least in Fas-induced apoptosis, other factors than AIF may be involved in the mediation of nuclear apoptosis. Further exploration of these poorly characterized in vitro systems will have to resolve this issue. As mentioned above, AIF fails to cleave nuclear PARP. Since the knock-out of the PARP gene has no major effects on apoptosis regulation, cleavage of this substrate is likely to constitute a consequence rather than a mechanism of apoptosis (36). However, since proteolysis of PARP is a common sign of nuclear apoptosis in cells (37) and in some cell-free systems of apoptosis (14, 25), additional (AIF-activated?) cytosolic proteases appear to be involved in the apoptotic degradation phase.

Mode of Action of Bcl-2. To unravel the relationship between AIF and Bcl-2, we have investigated three mutually non-exclusive possibilities. Bcl-2 might suppress (a) AIF synthesis; (b) AIF release; (c) or AIF action. Our data clearly indicate that Bcl-2-overexpressing mitochondria contain normal amounts of pre-formed AIF (Fig. 5, B and C), an observation that would be compatible with an eventual house-keeping function of this ubiquitous protein. Instead of preventing AIF formation (Fig. 5, B and C) or action (Figs. 6 and 7), Bcl-2 inhibits the PT-mediated release of AIF (Fig. 5, B and C). Our data suggest that Bcl-2 is primarily an inhibitor of PT (reference 4 and Fig. 5 A), in line with the facts that it is mainly situated in the inner-outer mitochondrial membrane contact sites (33, 34, 38) (where PT pores form [6]) and that its expression level correlates stoichiometrically with one of the putative PT pore constituents, the peripheral benzodiazepine receptor (39). Exploration of the mechanisms how Bcl-2 regulates PT-dependent AIF release will require further characterization of the PT pore complex.

In conclusion, Bcl-2 suppresses apoptotic PT and AIF release from mitochondria, yet does not interfere with the formation or action of AIF. This interpretation is in accord with previous genetic (40–43) and functional data (15, 43, 44), suggesting that, at least in some experimental systems, the mitochondrial but not the nuclear localization of Bcl-2 would determine its anti-apoptotic capacity. These results are also compatible with the absent or marginal apoptosis-

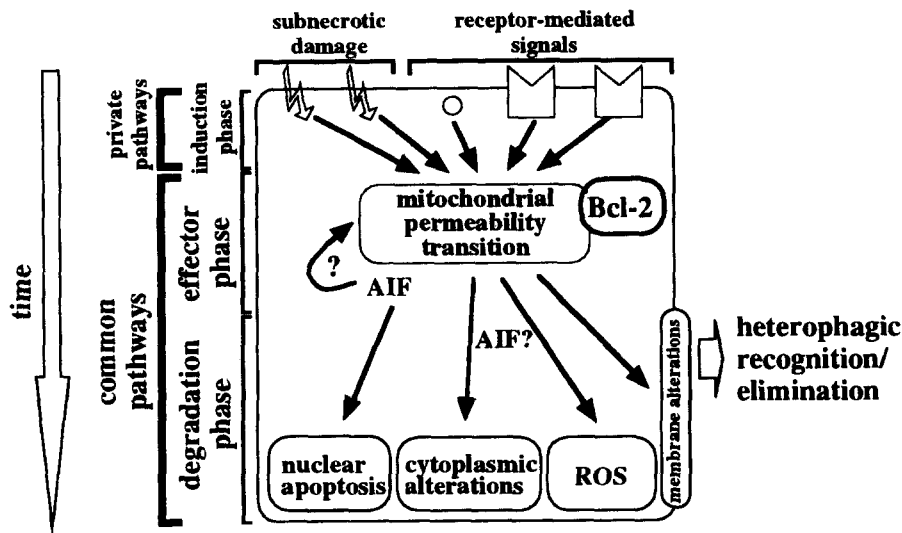


Figure 8. Hypothetical scenario of apoptosis regulation. The scheme provides an overview over the temporal and mechanistic advancement of the apoptotic process. Distinct apoptosis inducing pathways activated during the stimulus-dependent initiation phase of apoptosis converge at the level of the mitochondrion to induce permeability transition (PT), which is subjected to regulation by Bcl-2 and Bcl-2 homologues. PT and/or associated release of AIF triggers a number of functionally independent events: hypergeneration of reactive oxygen species, a number of cytoplasmic changes, nuclear apoptosis and apoptotic alterations of membrane structure. These latter are among the first consequences of PT, thus allowing for the heterophagic elimination of apoptotic cells by adjacent cells, before full-blown apoptosis occurs. At present it remains unknown which post-PT events are coordinated by AIF and whether AIF directly or indirectly engages in a self-amplifying PT-inducing feedback loop.

inhibitory effect of Bcl-2 on cell-free systems, when Bcl-2 protein is added to cytoplasmic extracts already containing soluble pro-apoptotic factors (15, 16).

Hierarchy between Bcl-2- and Protease-controlled Check Points of the Apoptotic Cascade. The present data may resolve a long-lasting controversy on the hierarchy between the putative control points of apoptosis regulated by Bcl-2/Ced-9 and ICE/Ced-3-like proteases, respectively (9–12, 31, 32). According to our data, Bcl-2 would prevent apoptosis by maintaining the subcellular compartmentalization of AIF, thereby suggesting a scenario that de facto abolishes the existence of such a hierarchy, at least at the level of the mitochondrial protease. However, it would be an oversimplification to assume that all proteases of the ICE/CPP32/Ced-3 family control the same point of the apoptotic cascade. Thus, ICE itself clearly acts upstream of the PT check point, in the sense that ICE activation triggered by Fas is an extremely rapid (<15–30 min) event (35) that precedes the PT-mediated $\Delta\Psi_m$ collapse. Inhibition of ICE by Ac-YVAD.cmk prevents the $\Delta\Psi_m$ disruption induced by Fas cross-linking (5). Moreover, recombinant ICE is a direct inducer of PT in isolated mitochondria (M. Castedo, S.A. Susin and G. Kroemer, manuscript in preparation). The protease inhibitors TLCK and Z-VAD.fmk can also prevent the glucocorticoid-triggered $\Delta\Psi_m$ disruption in thymocytes (8), suggesting the implication of one or several Z-VAD.fmk-inhibitable proteases in the induction of PT. When combined with the facts reported in this paper, this observation can be interpreted in two different ways. As a first possibility, one type of Z-VAD.fmk-inhibitable protease(s) would act upstream and another one, AIF, downstream of the mitochondrion. As a second possibility, AIF (and AIF-activated proteases) might engage in a self-amplification loop in which an initial triggering of PT causes AIF release, which in turn favors PT. These scenarios are currently under active investigation.

A Speculative Model of Apoptosis Regulation and Execution. Current models of apoptosis subdivide the common pathway into an initial effector phase, during which the “decision to die” is still subject to regulatory mechanisms, and a later degradation phase, beyond the “point-of-no-return”, during which the progressive augmentation of entropy due to the activation of catabolic enzymes precludes further regulation (3). Collectively, our data can be embedded into the following working hypothesis (Fig. 8). Different pathways of apoptosis induction (some of which may involve ROS production, ICE activation, changes in the expression level of Bcl-2/Bax etc.) converge at the level of the mitochondrion to induce PT and an associated $\Delta\Psi_m$ collapse. This event is controlled by Bcl-2 and probably by other Bcl-2 homologues located in the outer mitochondrial membrane. As a result of PT, which marks the point-of-no-return, the cell manifests multiple alterations: perturbation of the plasma membrane structure, increased production and action of superoxide anion, cytoplasmic changes, and nuclear apoptosis (2, 4, 8). At least some of these consequences of PT are dissociable among each other. Thus, the exposure of phosphatidylserine residues on the outer plasma membrane leaflet precedes hyperproduction of superoxide anions and does not require the presence of a nucleus (5); and nuclear apoptosis can be observed in ROS-free conditions (45). Hence, it appears that these manifestations of the degradation phase of apoptosis are not linked among each other via direct cause-effect relationships and are rather coordinated by a superior control event (8). Future investigations will have to determine to which extent the metabolic consequences of PT such as uncoupling of the respiratory chain or the PT-dependent AIF release function as coordinators of the apoptotic cascade.

In synthesis, the available data provide a plausible explanation for, as well as a number of testable hypotheses on the regulation of the apoptotic effector phase by proteases,

mitochondria, and proto-oncogenes of the Bcl-2 family. The progressive unmasking of the mechanism of action of Bcl-2, one of the major endogenous survival factors of

lymphoid cells and one of the clinically most important oncoproteins, may have far-reaching consequences for the rational design of apoptosis-modulatory drugs.

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