The Permeability Transition Pore Complex: A Target for Apoptosis Regulation by Caspases and Bcl-2-related Proteins

By Isabel Marzo,* Catherine Brenner,* Naoufal Zamzami,* Santos A. Susin,* Gisela Beutner,† Dieter Brdiczka,‡ René Rémy,§ Zhi-Hua Xie,‖ John C. Reed,‖ and Guido Kroemer*†

From the *Centre National de la Recherche Scientifique, Unité Propre de Recherche 420, F-94801 Villejuif, France; the †Faculty of Biology, University of Konstanz, D-78434 Konstanz, Germany; the ‡Centre National de la Recherche Scientifique, Université de Paris 11, F-91405 Orsay, France; and ‖The Burnham Institute, La Jolla, California 92037

Summary

Early in programmed cell death (apoptosis), mitochondrial membrane permeability increases. This is at least in part due to opening of the permeability transition (PT) pore, a multiprotein complex built up at the contact site between the inner and the outer mitochondrial membranes. The PT pore has been previously implicated in clinically relevant massive cell death induced by toxins, anoxia, reactive oxygen species, and calcium overload. Here we show that PT pore complexes reconstituted in liposomes exhibit a functional behavior comparable with that of the natural PT pore present in intact mitochondria. The PT pore complex is regulated by thiol-reactive agents, calcium, cyclophilin D ligands (cyclosporin A and a nonimmunosuppressive cyclosporin A derivative), ligands of the adenine nucleotide translocator, apoptosis-related endoproteases (caspases), and Bcl-2-like proteins. Although calcium, prooxidants, and several recombinant caspases (caspases 1, 2, 3, 4, and 6) enhance the permeability of PT pore-containing liposomes, recombinant Bcl-2 or Bcl-XL augment the resistance of the reconstituted PT pore complex to pore opening. Mutated Bcl-2 proteins that have lost their cytoprotective potential also lose their PT modulatory capacity. In conclusion, the PT pore complex may constitute a crossroad of apoptosis regulation by caspases and members of the Bcl-2 family.

Two different major changes in mitochondrial membrane permeability have been observed during the effector phase of apoptosis. On the one hand, the electrochemical gradient built up on the mitochondrial inner membrane dissipates early during apoptosis (1–4). On the other hand, apoptogenic proteins that normally are sequestered in mitochondria are released via the outer mitochondrial membrane. Such proteins include cytochrome c (5–7) and apoptosis inducing factor (AIF)1 (8, 9). The protooncogene product Bcl-2 prevents the permeability increase in both mitochondrial membranes (4, 6–10). Based on the similarity of the effects of Bcl-2 and pharmacological inhibitors of the mitochondrial permeability transition (PT) pore, we have advanced the hypothesis that opening of the PT pore might be (co-)responsible for the apoptosis-associated changes in mitochondrial membrane function (2, 4, 8, 11). In isolated mitochondria, opening of the PT pore entails both the disruption of the inner mitochondrial transmembrane potential (∆Ψm) (12, 13) and the release of the apoptogenic proteins AIF (8, 9) and cytochrome c (14, 15), suggesting that the PT pore may have an important role in cell death control. Moreover, opening of the PT pore has been implicated in clinically relevant massive cell death of hepatocytes, neurons, and myocardiocytes induced by hepatotoxins, excitotoxins, calcium, reactive oxygen species, and anoxia (3, 4, 12, 13, 16–18 and references cited therein).

If the mitochondrial fulfilled a major role in apoptosis control, it should be capable of integrating very different proapoptotic signal transduction and damage pathways. In this context, it appears important that the PT pore is a dynamic multiprotein complex located at the contact site between the inner and the outer mitochondrial membranes, one of the critical sites of metabolic coordination between the cytosol, the mitochondrial intermembrane space, and...
the matrix. The PT pore participates in the regulation of matrix Ca\(^{2+}\), pH, \(\Delta_{\text{mem}}\), and volume and functions as a Ca\(^{2+}\)-, voltage-, pH-, and redox-gated channel with several levels of conductance and little if any ion selectivity (12, 13, 19). Although the exact composition of the PT pore complex (PTPC) is unknown, it is thought to involve proteins from the cytosol (hexokinase), the outer membrane (voltage-dependent anion channel [VDAC]), the inner membrane (the adenine nucleotide translocator [ANT]), and the matrix (cyclophilin D) (12, 13, 20–23). As a consequence, the PT pore complex contains multiple targets for endogenous regulators. In intact cells and isolated mitochondria, PT pore opening is induced by several proapoptotic signals: Ca\(^{2+}\), prooxidants, nitric oxide, ceramide, and caspase 1 (1, 2, 8, 9, 12, 13, 19, 24–27).

Moreover, it is regulated by the antiapoptotic oncoproteins Bcl-2, Bcl-X\(_L\), Bcl-W, and Bcl-\(\text{Dc}\), as well as the proapoptotic Bcl-\(\text{D}\), which stabilize mitochondrial membranes (4, 8, 9, 28–31), and by the proapoptotic Bcl-2 analogue Bax, which disrupts the \(\Delta_{\text{mem}}\) (32).

It has been unclear whether these effectors specifically act on PTPC, affect other mitochondrial structures not associated with PTPC (6, 7), or rather nonspecifically perturb membrane permeability, as this has been suggested for members of the Bcl-2 family (32–35). To distinguish these possibilities, we purified protein complexes containing PTPC, reconstituted them in liposomes, and created a reduced experimental system that shares properties of the PT pore studied in intact mitochondria or cells. Biochemical and functional data indicate that PTPC enriched from brain homogenates contain the proapoptotic Bcl-2 homologue Bax (but not Bcl-2 and Bcl-X\(_L\)), in addition to proteins previously suggested to participate in the regulation of PT (ANT, VDAC, cyclophilin D, and hexokinase). The membrane permeability of PTPC liposomes was enhanced by several inducers of PT including Ca\(^{2+}\), prooxidants, and recombinant caspases. R recombinant Bcl-2 and Bcl-X\(_L\), as well as inhibitors of PT pore opening in this artificial system. Thus, PTPC constitutes the target of multiple apoptosis regulators, emphasizing its probable central role in cell death control.

Materials and Methods

Materials. R recombinant human Bcl-X\(_L\) (1–209), Bcl-2 (1–218), mutant Bcl-2 (Gly145Ala), and Bcl-2\(\Delta_{\text{a5/6}}\) (\(\Delta_{143–184}\)), all lacking the hydrophobic transmembrane domain (\(\Delta_{219–239}\) in the case of Bcl-2; \(\Delta_{210–230}\) for Bcl-X\(_L\)) and tagged NH\(_2\) terminal with His\(_8\), were produced and purified as described (34). R recombinant caspases were produced as active enzymes (36, 37). Caspase activity is defined as amount of enzyme required to cleave 1 mol of the fluorogenic substrate Ac-DEVD.amc (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; caspases 3 and 6), Ac-YVAD.amc (acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin; caspase 1), or Ac-WEHD.amc (acetyl-Val-Ile-Pro-Arg-aminomethylcoumarin; caspase 8) per hour. Caspase substrates and inhibitors (Ac-DEVD.cmk [acetyl-Asp-Glu-Val-Asp-aminomethylketone], Ac-YVAD.cmk [acetyl-Tyr-Val-Ala-Asp-aminomethylketone]) were purchased from Bachem (Basel, Switzerland). All remaining reagents were from Sigma Chemical Co. (St. Louis, MO), unless specified differently.

Reconstitution of PTPCs in Liposomes. PTPCs were purified and reconstituted in liposomes following published protocols (22), with several modifications (Fig. 1A). In brief, four Wistar (Philadelphia, PA) rat brains (3–4-mo-old males, stored at \(-80^\circ\text{C}\)) were homogenized in buffer 1 (1 mM \(\alpha\)-monothioglycerol, 10 mM glucose, pH 8.0, 40 ml; sample 1 in Fig. 1) and centrifuged twice (15 min, 12,000 g. 4°C) to resuspend the pellet first in buffer 1 alone, and then in buffer 1 plus 0.5% Triton X-100 (Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature (RT) while stirring. Supernatants (40 min, 50,000 g, 4°C) of this mixture, the Triton-soluble protein fraction (sample 2 in Fig. 1), were mixed with 17 g DE52 resin previously equilibrated with buffer 2 (1.5 mM Na\(_2\)HPO\(_4\), 1.5 mM K\(_2\)HPO\(_4\), 100 mM glucose, 1 mM dithioerythritol, pH 8.0). These beads were packed into an FPLC column (XK16/20; Pharmacia Biotech, Uppsala, Sweden) and eluted with buffer 2 supplemented with 50 mM KC1 (buffer 3) or 400 mM KC1 (buffer 4). For equilibration with buffer 3 (0.8 ml/min, 6 ml), elution was performed on a linear gradient from 50 to 400 mM KC1 (buffers 3 versus 4), followed by determination of hexokinase activity (sample 3 in Fig. 1). Lipid vesicles were prepared by mixing 300 mg phosphatidylcholine and 60 mg cholesterol in 3 ml chloroform, evaporation of the chloroform under nitrogen, and resuspension in 3 ml 125 mM sucrose \(+10\) mM Hepes (pH 7.4) + 0.3% n-octyl-\(\beta\)-d-pyranoside by vortexing (90 min, RT). These vesicles (6 ml) were incubated with 6 ml of PTPC-containing solution during 20 min at RT and dialyzed overnight (4°C) against 125 mM sucrose \(+10\) mM Hepes (pH 7.4). In several experiments, recombinant Bcl-2, Bcl-X\(_L\), or mutated Bcl-2 proteins were added during the dialysis step at a dose corresponding to 5% of the total PTPC proteins as determined in each experiment. Liposomes recovered from dialysis were ultrasonicated (120 W, Ultrasonic Processor; Bioblock, Illkirch, France) during 7 s in 5 mM maltate and 10 mM KC1, charged on a Sephadex G50 column (C16/20; Pharmacia Biotech, Inc., Eugene, OR), and eluted with 125 mM sucrose \(+10\) mM Hepes (pH 7.4, 0.8 ml/min) (Fig. 1C). Proteins were extracted from the liposome preparation (1 ml) by mixing with 2 ml 880 ml KCl \(+6\) ml chloroform/methanol (2:1 vol/vol) and recovered from the interface after standard methods (38), followed by resuspension in 0.1% SDS (sample 4 in Fig. 1). They were then precipitated with 80% (vol/vol) acetone for two-dimensional electrophoresis. A mean of 1.86 \pm 0.24 \mu g protein/mg lipid (X \(= SD, n = 5\)) was recovered from proteoliposomes. In several experiments, purified rat cytochrome c (25 \mu g/ml, corresponding to 500 ng cytochrome c/ml lipid) was added before the sonication step, followed by two washes on Sephadex G50 columns to remove excess cytochrome c from the supernatant.

Determination of Ca\(^{2+}\) Efflux from PTPC Liposomes. Liposomes were generated as described above with the sole difference that sonication was performed in 8 mM calcium (Molecular Probes Inc., Eugene, OR) \(+10\) mM cobalt chloride. 200 \mu l liposome suspension was incubated for 90 min with different concentration of atracysolide (Atr) and/or 50 \mu M bongkrekic acid (gift from H. J. Duine, Hans J. Duine, Delft University, Delft, The Netherlands). The supernatants of liposomes (4.5 g, 45 min, 4°C) were recovered, supplemented with EDTA (final concentration of 1 mM), and subjected to fluorometric analysis (excitation at 488 nm, emission at 520 nm) in a fluorescence spectrometer (F4500; Hitachi, Tokyo, Japan).

Western Blots and Two-dimensional Electrophoresis. Total brain homogenates, Triton-soluble proteins, PTPC preparations from anion exchange columns, and proteins extracted from PTPC-reconstituted liposomes were separated by SDS-PAGE (10–15%, 30 \mu g
protein/lane), followed by Western blot using monoclonal antibodies recognizing cytochrome c (PharMingen, San Diego, CA; reference 5), hsp60 (clone LK1; Sigma Chemical Co.), VDAC (gift from F.Thinnes, Molecular Pathology Institute for Experimental Medicine, Göttingen, Germany), or polyclonal rabbit antibodies recognizing cytochrome b oxidase (gift from T. Wallimann, Zürich, Switzerland). The more abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite YL510; Polyscience, Warrington, PA) of defined diameters was used for the analysis of protein/lane. The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 500 to 8000, respectively. The fluoromicroscopy performed the presence of mainly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were incubated during 15 min at 37°C in the presence of the indicated dose of active or inhibitor-inactivated caspases. Diluted (1 ml) liposomes were incubated with 3,3'-dihexylocarbocyanine iodide (DiOC 6(3), 80 nM, 20–30 min at RT; Molecular Probes), followed by analysis of DiOC 6(3) retention in a FACScan Vantage cytofluorometer (Becton Dickinson, San José, CA). The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 1500 events/s. The photomultiplier of the side scatter and FL1 were set at 700 mV and 700–800 mV, respectively. The fluorescence was excited with an Argon laser (excitation wavelength 488 nm) and analyzed in FL-1 (wave length 530 ± 30 nm). The forward and side scatters were gated on the quantitatively most abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite BB; Polyscience, Warrington, PA) of defined diameters was used to determine the diameter of liposomes that were gated on (gate: 150 to 300 nm; mean size of liposomes 230 ± 60 nm; X ± SD for 5 \times 10^4 events). Electron microscopy confirmed the presence of mostly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were analyzed for each data point. Results are expressed as percent of reduction of DiOC 6(3) fluorescence (log scale, geometric mean), considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Evaluation of Caspase Effects on Isolated Mitochondria. Purified mouse liver mitochondria were incubated in 10 mM Tris-MOPS buffer proteins ([Molecular Probes]). Alternatively, liposomes were incubated during 15 min at 37°C in the presence of the indicated dose of active or inhibitor-inactivated caspases. Diluted (1 ml) liposomes were incubated with 3,3'-dihexylocarbocyanine iodide (DiOC 6(3), 80 nM, 20–30 min at RT; Molecular Probes), followed by analysis of DiOC 6(3) retention in a FACScan Vantage cytofluorometer (Becton Dickinson, San José, CA). The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 1500 events/s. The photomultiplier of the side scatter and FL1 were set at 700 mV and 700–800 mV, respectively. The fluorescence was excited with an Argon laser (excitation wavelength 488 nm) and analyzed in FL-1 (wave length 530 ± 30 nm). The forward and side scatters were gated on the quantitatively most abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite BB; Polyscience, Warrington, PA) of defined diameters was used to determine the diameter of liposomes that were gated on (gate: 150 to 300 nm; mean size of liposomes 230 ± 60 nm; X ± SD for 5 \times 10^4 events). Electron microscopy confirmed the presence of mostly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were analyzed for each data point. Results are expressed as percent of reduction of DiOC 6(3) fluorescence (log scale, geometric mean), considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Evaluation of Caspase Effects on Isolated Mitochondria. Purified mouse liver mitochondria were incubated in 10 mM Tris-MOPS buffer proteins ([Molecular Probes]). Alternatively, liposomes were incubated during 15 min at 37°C in the presence of the indicated dose of active or inhibitor-inactivated caspases. Diluted (1 ml) liposomes were incubated with 3,3'-dihexylocarbocyanine iodide (DiOC 6(3), 80 nM, 20–30 min at RT; Molecular Probes), followed by analysis of DiOC 6(3) retention in a FACScan Vantage cytofluorometer (Becton Dickinson, San José, CA). The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 1500 events/s. The photomultiplier of the side scatter and FL1 were set at 700 mV and 700–800 mV, respectively. The fluorescence was excited with an Argon laser (excitation wavelength 488 nm) and analyzed in FL-1 (wave length 530 ± 30 nm). The forward and side scatters were gated on the quantitatively most abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite BB; Polyscience, Warrington, PA) of defined diameters was used to determine the diameter of liposomes that were gated on (gate: 150 to 300 nm; mean size of liposomes 230 ± 60 nm; X ± SD for 5 \times 10^4 events). Electron microscopy confirmed the presence of mostly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were analyzed for each data point. Results are expressed as percent of reduction of DiOC 6(3) fluorescence (log scale, geometric mean), considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Evaluation of Caspase Effects on Isolated Mitochondria. Purified mouse liver mitochondria were incubated in 10 mM Tris-MOPS buffer proteins ([Molecular Probes]). Alternatively, liposomes were incubated during 15 min at 37°C in the presence of the indicated dose of active or inhibitor-inactivated caspases. Diluted (1 ml) liposomes were incubated with 3,3'-dihexylocarbocyanine iodide (DiOC 6(3), 80 nM, 20–30 min at RT; Molecular Probes), followed by analysis of DiOC 6(3) retention in a FACScan Vantage cytofluorometer (Becton Dickinson, San José, CA). The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 1500 events/s. The photomultiplier of the side scatter and FL1 were set at 700 mV and 700–800 mV, respectively. The fluorescence was excited with an Argon laser (excitation wavelength 488 nm) and analyzed in FL-1 (wave length 530 ± 30 nm). The forward and side scatters were gated on the quantitatively most abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite BB; Polyscience, Warrington, PA) of defined diameters was used to determine the diameter of liposomes that were gated on (gate: 150 to 300 nm; mean size of liposomes 230 ± 60 nm; X ± SD for 5 \times 10^4 events). Electron microscopy confirmed the presence of mostly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were analyzed for each data point. Results are expressed as percent of reduction of DiOC 6(3) fluorescence (log scale, geometric mean), considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Evaluation of Caspase Effects on Isolated Mitochondria. Purified mouse liver mitochondria were incubated in 10 mM Tris-MOPS buffer proteins ([Molecular Probes]). Alternatively, liposomes were incubated during 15 min at 37°C in the presence of the indicated dose of active or inhibitor-inactivated caspases. Diluted (1 ml) liposomes were incubated with 3,3'-dihexylocarbocyanine iodide (DiOC 6(3), 80 nM, 20–30 min at RT; Molecular Probes), followed by analysis of DiOC 6(3) retention in a FACScan Vantage cytofluorometer (Becton Dickinson, San José, CA). The forward scatter threshold was set at 30 (Amp 16) and the flow-rate at 1500 events/s. The photomultiplier of the side scatter and FL1 were set at 700 mV and 700–800 mV, respectively. The fluorescence was excited with an Argon laser (excitation wavelength 488 nm) and analyzed in FL-1 (wave length 530 ± 30 nm). The forward and side scatters were gated on the quantitatively most abundant population of liposomes while excluding background noise. Calibration with carboxylate microspheres (Fluoresbrite BB; Polyscience, Warrington, PA) of defined diameters was used to determine the diameter of liposomes that were gated on (gate: 150 to 300 nm; mean size of liposomes 230 ± 60 nm; X ± SD for 5 \times 10^4 events). Electron microscopy confirmed the presence of mostly unilamellar proteoliposomes of the expected size in the PTPC liposome preparation. Triplicates of 5 \times 10^4 liposomes were analyzed for each data point. Results are expressed as percent of reduction of DiOC 6(3) fluorescence (log scale, geometric mean), considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.
+ 100 mM NH₄Cl + 10 μM EGTA (pH 7.2) during 30 min at RT in the presence of different caspases. The supernatant (1.5 × 10⁵ g) of these mitochondria was stored at −80°C until testing for apoptogenic activity on isolated HeLa nuclei (90 min, 37°C, RT). DNA fragmentation was quantified by propidium iodine staining (10 μg/ml, ≥5 min at RT) and cytofluorometric analysis in an EPICS Prolife II (Coulter, Hialeah, FL), as described (26). Results were expressed as the percentage of subdiploid nuclei, after subtraction of values obtained with buffer only (<20%). For control purposes, different caspase inhibitors (Ac-DEVD.cmk, Ac-YVAD.cmk, or Z-VAD.fmk (N-benzoylxyacarbonyl-Val-Ala-Asp-fluoromethylketone); 100 μM final concentration) were added to the mitochondrial supernatant 15 min before determination of apoptogenic activity. Aliquots of caspase-treated mitochondria were resuspended in 400 mM mannitol, 50 mM Tris (HCl, pH 7.2), 5 mg/ml BSA, 10 mM KH₂PO₄, and 5 mM succinate, and then labeled with DiOC₆(3) (100 nM, 15 min at RT) and subjected to cytofluorometric analysis using carbonylcyanide m-chlorophenylhydrazone (CCCP; 50 μM) or Atr (5 mM) as positive controls of maximum ΔΨₘ disruption.

Results

Reconstitution of the PTPC in Liposomes. Hexokinase 1 is a cytosolic protein, part of which associates with the mitochondrial outer membrane where it binds to porin within the contact site (22, 40–42). Taking advantage of this fact, we traced the hexokinase activity copurifying with a protein complex that is water insoluble in brain homogenates, partitions into the triton-soluble fraction, elutes from an anion exchange FPLC column at a relatively high salinity, and incorporates into phosphatidylincholine cholesterol liposomes (Fig. 1 A–C). When comparing the abundance of proteins extracted from liposomes incorporating hexokinase activity with that of the preceding purification steps, it appears that some proteins are selectively enriched (cyclophilin D, the ~60-kD isoform of the ANT, Bax, Bag-1), whereas some are reduced (VDAC, F₁ ATPase) or eliminated below the limit of detection (Bcl-X₁, Bcl-2, Bad, cytochrome c, hsp60; Fig. 1 D). As shown by two-dimensional gel electrophoresis, PTPC liposomes contain a limited set of proteins whose complete identification is still in progress (Fig. 2). PTPC-containing liposomes can be treated with inducers of PT pore opening, which cause the release of encapsulated molecules such as malate (106 daltons) and ATP (509 daltons) (22, 43). Similarly, the fluorochrome calcine (622 daltons), a hydrophilic polyanionic fluorochrome previously used to measure PT pore opening in intact cells (44), can be encapsulated into PTPC liposomes and then released by incubation with the ANT ligand Atr, a potent inducer of PT pore opening (Fig. 3 A). This effect is prevented by another ANT ligand, bongkrekic acid, which inhibits PT pore opening (Fig. 3 A). We have developed another approach to quantify PT pore opening induced in PTPC liposomes. Liposomes were equilibrated with the amphophilic cationic fluorochrome DiOC₆(3) (573 daltons) and then labeled with DiOC₆(3) (100 nM, 15 min at RT) and subjected to cytofluorometric analysis using carbonylcyanide m-chlorophenylhydrazone (CCCP, 50 μM) or Atr (5 mM) as positive controls of maximum ΔΨₘ disruption.

Figure 2. Two-dimensional gel electrophoresis of proteins extracted from PTPC liposomes (Fig. 1 A, 4). Silver-stained proteins whose abundance is consistently (three experiments) reduced upon digestion with caspase 1 (1.5 U/ml) are marked in rectangles. Results are representative for three independent experiments.
groups (target of diamide and monochlorobiman), as well as Ca$^{2+}$-sensitive sites, participate in the regulation of the PTPC (12, 13, 20–23).

**Effect of Recombinant Bcl-2 and Bcl-XL on PTPC.** Under the conditions of fractionation described in Fig. 1, a proapoptotic member of the Bcl-2 family (Bax) selectively coenriches with components of PTPC, whereas several antiapoptotic members of the Bcl-2 family do not (Bcl-XL, Bcl-2; Fig. 1 D). We therefore investigated the effect of antiapoptotic members of the Bcl-2 family on PTPC. Recombinant Bcl-2 and Bcl-XL proteins, as well as mutant Bcl-2 proteins, were incorporated into liposomes together with PTPC via dialysis, a procedure that allows for the oriented, pH-independent incorporation of proteins into lipid membranes (45, 46). Irrespective of the presence of Bcl-2-like proteins, all liposome preparations consistently (n = 12) maintained a similar baseline DiOC$_6$(3) fluorescence (mean fluorescence channel $620 \pm 18$ and $616 \pm 19$ in the presence or absence of Bcl-2, respectively, X $\pm$ SEM, 12 independent experiments), with comparable SDS-releasable DiOC$_6$(3) release (Fig. 4 A) for as long as 8 h (not shown), suggesting that Bcl-2 does not augment the membrane permeability in this experimental system. Moreover, Bcl-2 does not perturb the ultrastructure of PTPC liposomes or their protein composition (not shown). The presence of Bcl-XL, or Bcl-2 protected against the DiOC$_6$(3) release induced by atractyloside, tert-butylhydroperoxide, as well as low doses of Ca$^{2+}$, but not by diamide (Fig. 4 C). Similar results were obtained, when instead of DiOC$_6$(3) retention, calcine efflux was studied (not shown). These effects correlate with the functional potency of Bcl-2, which protects cells against most PT inducers (8, 9, 47), but not against diamide (8, 9). A Bcl-2 deletion mutant lacking a putative channel-forming domain corresponding to the $\alpha_5$ and $\alpha_6$ helices, Bcl-2$\Delta\alpha_5\alpha_6$, which has lost its antiapoptotic function (34), failed to prevent the DiOC$_6$(3) release. In addition, a Bcl-2 point mutant in the BH1 region, Bcl-2(Gly145Ala), which does not interact with Bax, failed to protect against apoptosis (48) and had no inhibitory effect on PTPC liposomes (Fig. 4 C). Altogether, these findings suggest that Bcl-2 can regulate membrane permeability by acting on or in concert with PTPC.

**Effect of Recombinant Caspases on PTPC.** Since caspases are involved at all stages of apoptosis (5, 26, 49–51), we tested whether caspases might act on PTPC. PTPC reconstituted in liposomes were exposed to recombinant caspases, fol-
and 6) abolish caspase-induced DiOC6(3) release, suggesting for caspases 1 and 4 and Ac-DEVD.cmk for caspases 2, 3, shown). Tetrapeptide inhibitors of caspases (Ac-YVAD.cmk containing liposomes, but not in control liposomes (not 

Discussion

Failure of PTPC to Release Cytochrome c. Since induction of PTPC in intact mitochondria causes cytochrome c release (14, 15; and unpublished data), and since several groups have suggested that Bcl-2 primarily regulates the release of cytochrome c via the outer mitochondrial membrane rather than PT (6, 7, 31, 52), we investigated the putative relationship between PT pore opening and cytochrome c. Incorporation of purified cytochrome c into PTPC liposomes (which constitutively are devoid of cytochrome c, Fig. 1 D) does not alter their functional behavior. Thus, PTPC liposomes containing cytochrome c exhibit a normal baseline level of DiOC6(3) retention and release DiOC6(3) in response to Atr and caspases in a Bcl-2–inhibitable fashion (Fig. 7 A). Although such liposomes contain significant amounts of SDS-releasable cytochrome c, they fully retain cytochrome c when incubated with doses of Atr or caspase that cause DiOC6(3) release (Fig. 7 B). This indicates that PTPC are not directly responsible for the release of cytochrome c.
Inhibitors (which inhibit caspases but not AIF) or in the presence of Z-VAD.fmk (which inhibits AIF) to exclude that nuclear DNA degradation is a direct caspase effect. Similar results were obtained with mouse and rat (not shown) hepatocyte mitochondria.

Figure 5. Effect of caspases on PT-P C liposomes and isolated mitochondria. (A) Representative DiOC₆(3) fluorescence histograms obtained after treatment of liposomes with various caspases (1.2 U/ml for caspase 1, 10 U/ml for caspase 6) in the presence or absence of the indicated caspase inhibitor (100 μM). (B) Dose dependency of effects obtained with different recombinant caspases on PT-P C liposomes. (C) Effect of caspases on the Δψm. Mitochondria were treated during 30 min with 5 U caspase/200 μl, followed by determination of the Δψm using DiOC₆(3). The protonophore m-chlorophenylhydrazine (50 μM) defined 100% Δψm disruption. (D) Release of AIF into the mitochondrial supernatant. Intact mitochondria were treated with the indicated caspase (5 U/200 μl), followed by centrifugation and removal of the supernatant that was tested for apoptogenic activity on isolated HeLa nuclei. The incubation was performed in the presence of tetrapeptide inhibitors (which inhibit caspases but not AIF) or in the presence of Z-VAD.fmk (which inhibits AIF) to exclude that nuclear DNA degradation is a direct caspase effect. Similar results were obtained with mouse and rat (not shown) hepatocyte mitochondria.

Figure 6. Effect of Bcl-2 on the caspase induced DiOC₆(3) release observed in PT-P C liposomes. (A) Representative DiOC₆(3) staining profiles. Liposomes were generated in the presence of recombinant Bcl-2, Bcl-XL, and the indicated Bcl-2 mutants, treated with 1 U caspase 1, and labeled with DiOC₆(3) to determine the DiOC₆(3) release. Results are representative for at least three independent determinations. (B) Dose response curves of caspase effects on liposomes containing Bcl-XL, Bcl-2, or Bcl-2 mutants.
mitochondria. Thus, using a number of different inducers and inhibitors of PT, we found an approximate functional equivalence between the natural (mitochondrial) PTPC and the reconstituted (liposomal) PTPC (Fig. 3) in the regulation of membrane permeability. Both in mitochondria and in PTPC liposomes, a similar panel of agents acts to permeabilize membranes (Ca\(^{2+}\), Atr, prooxidants, and diamide) or to stabilize membrane function (cyclosporin A, monochlorobiman, and bongkrekic acid; references 8, 12, 13). This suggests, in line with previous observations (5, 26, 30, 55–58) or, on the contrary, efficiently counteract caspase 1–mediated apoptosis (30, 59, 60). Moreover, the fact that Bcl-2 mitogates the PT induced by caspases that are broadly involved in apoptosis (e.g., caspases 3 and 6) suggests that it can interrupt a self-amplifying loop in which caspase effects on mitochondria favor the release of caspase activators. We have investigated whether Bcl-2 acts as an inhibitor of caspase-mediated digestion of PTPC proteins. Our preliminary findings indicate that Bcl-2 does not prevent the digestion of caspase 1 substrates (not shown), suggesting that it inhibits the functional consequence of caspase 1–mediated proteolysis rather than proteolysis itself. It has been shown recently that caspase 3 cleaves Bcl-2, thereby converting it from a death inhibitor to a death promoter (61). However, caspase 1 does not digest Bcl-2, at least in the conditions reported in Fig. 6 A, suggesting the functional relevance of additional caspase targets within the PTPC.

Crystallographic data (62) and studies of artificial membranes containing Bcl-X\(_L\) or Bcl-2 (33, 34) suggest that Bcl-2–like proteins constitute ion channels. However, Bcl-X\(_L\) and Bcl-2 incorporated into membranes containing PTPC, rather than increasing membrane permeability, stabilize PTPC liposomes and prevent PT pore opening. This apparent discrepancy may be explained by the composition...
of the artificial membranes, which only allow Bcl-2 to form channels when they contain, in addition to neutral lipids (as in this paper), an unusually high percentage (30–40%) of acidic lipids (33, 34). At present, we cannot discriminate between the possibilities that the PT-inhibitory effect of Bcl-2 is due to interactions with and conformational effects on PTPC constituents, or rather due to the specific neutralization of Bax (35, 48), a proapoptotic molecule that is present in PTPC (Fig. 1 D) and favors PT (32). Irrespective of these possibilities, the Bcl-2 effect on PTPC correlates with its antiapoptotic potential in the sense that mutations or deletions abolishing the death antagonistic correlates with its antiapoptotic potential in the sense that mutations or deletions abolishing the death antagonistic potential of Bcl-2 also abrogate its PT-inhibitory function.

In addition to its PT-inhibitory effect, which may account for at least part of its cytoprotective action, Bcl-2 has further pleiotropic effects (4, 10). Although some of these effects, including those concerning the capacity of Bcl-2 to affect redox regulation or intracellular Ca\(^{2+}\) partition, may be secondary to PT modulation; others are more difficult to accommodate in a model in which the major action of Bcl-2 would be PT regulation. This applies, in particular, to the participation of Bcl-2 participation in a multiprotein ensemble or “apoptosome” involving the mammalian CED-4 homologue(s), cytochrome c, and large prodomain caspases. As a possibility, Bcl-2 could exert a dual function in which it simultaneously or sequentially acts on PTPC and inactivates the apoptosome (10).

The Central Executioner of Apoptosis: Involvement of PTPC? Changes in mitochondrial membrane function have been proposed to form part of the “central executioner” (63), colloquially also referred to as “great integrator” or “apostat” (2, 3, 6–8, 11). Activation of the central executioner during the effector stage would control the commitment to undergo cell death and unify the many private induction pathways of apoptosis into one common pathway. The findings reported herein indicate that PTPC can constitute a crossroad at which physiological modulators of PT (Ca\(^{2+}\), Mg\(^{2+}\), pH, ADP, ATP, NAD(P)H, glutathione, ceramide, lipid oxidation products, etc.; references 12, 13, 19; Fig. 3), caspases (Fig. 5), and Bcl-2 homologues (Fig. 4, 6) together influence the fate of the cell. Thus, PTPC may simultaneously collect information on the metabolic stage of the cell, signal transduction pathways, as well as on the composition of the Bcl-2 complex. Opening of the PT pore, which occurs almost universally during apoptosis, has lethal repercussions including the mitochondrial generation of reactive oxygen species, disruption of oxidative phosphorylation, and the mitochondrial release of apoptogenic proteins necessary for the activation of downstream caspases and endonuclease activation (1–10, 14, 15).

In conclusion, PTPC may be identical with or form part of the critical structure that integrates different apoptosis induction pathways, decides the fate of the cell, and coordinates the common death program. If this interpretation is correct, the future elucidation of the exact composition and fine tuning of PTPC should furnish invaluable clues to the understanding of the apoptotic process.

We thank Drs. A. Srinivasan and K. Tomaseilli (Idun Pharmaceuticals, La Jolla, CA) for recombinant caspases 1, 3, and 6; N. Thornberry (Merck, Rahway, NJ) for caspases 1, 2, and 4; G. Salvesen (The Burnham Institute, La Jolla, CA) for caspases 3 and 6; Drs. S. Matsujama, C. Aimé-Sempé, and S. Takajama (The Burnham Institute) for Bcl-2 plasmid constructions. Electron microscopic analyses of PTPC liposomes were performed by Marie-Christine Prévost (Pasteur Institute, Paris, France).

This work has been supported by grants from the Agence Nationale pour la Recherche contre le SIDA; Association pour la Recherche contre le Cancer, Centre National de la Recherche Scientifique, Fondation pour la Recherche Médicale, Institut National de la Santé et de la Recherche Médicale, Ligue Nationale contre le Cancer (to G. Kroemer), University of California Breast Cancer Research Program (grant No. IRB-0098) and CalP-CURE Inc. (to J.C. Reed). I. Marzo and S.A. Susin receive fellowships from the Spanish Ministry of Science and from the European Commission, respectively.

Address correspondence to Guido Kroemer, 19 rue Guy Môquet, B.P. 8, F-94801 Villejuif, France. Phone: 33-1-49-58-35-13; Fax: 33-1-49-58-35-09; E-mail: kroemer@infobiogen.fr

Received for publication 22 October 1997 and in revised form 13 January 1998.

References
1. Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J.-L. Vayssière, P.X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J. Exp. Med. 181:1661–1672.
2. Zamzami, N., P. Marchetti, M. Castedo, D. Decaudin, A. Macho, T. Hirsch, S.A. Susin, P.X. Petit, B. Mignotte, and G. Kroemer. 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. 182:367–377.
3. Kroemer, G., N. Zamzami, and S.A. Susin. 1997. Mitochondrial control of apoptosis. Immunol. Today. 18:44–51.
4. Kroemer, G. 1997. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. 3:614–620.
5. Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts requirement for dATP and cytochrome c. Cell. 86:147–157.
6. Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Caí, T.-I. Peng, D.P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Biochem. Biophys. Res. Commun. 232:669–671.

7. Kluck, R.M., E. Bossy-Wetzel, D.R. Green, and D.D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 275:1132–1136.

8. Zamzami, N., S.A. Susin, P. Marchetti, T. Hirsch, I. Gómez-Monterrey, M. Casteño, and G. Kroemer. 1996. Mitochondrial control of nuclear apoptosis. J. Exp. Med. 183:1533–1544.

9. Susin, S.A., N. Zamzami, M. Casteño, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184:1331–1342.

10. Reed, J.C. 1997. Double identity for proteins of the Bcl-2 family. Nat. Rev. 387:773–776.

11. Marchetti, P., M. Casteño, S.A. Susin, N. Zamzami, T. Hirsch, A. Haeffner, F. Hirsch, M. Geuskens, and G. Kroemer. 1996. Mitochondrial permeability transition is a central coordinating event of apoptosis. J. Exp. Med. 184:1155–1160.

12. Zoratti, M., and I. Szabó. 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta Rev. Biomembranes. 1241:139–176.

13. Bernardi, P., and V. Petronilli. 1996. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J. Bioenerg. Biomembr. 28:129–136.

14. Kantrow, S.P., and C.A. Piantadosi. 1997. The release of cytochrome c from liver mitochondria during permeability transition. Biochim. Biophys. Res. Commun. 232:669–671.

15. Ellerby, H.M., S.J. Martin, L.M. Ellerby, S.S. Naïem, S. Rabizadeh, G.S. Salvese, C.A. Caiano, N.R. Cashman, D.R. Green, and D.E. Bredesen. 1997. Establishment of a cell-free system of neuronal apoptosis comparison of premitochondrial, mitochondrial, and postmitochondrial phases. J. Neurosci. 17:6165–6178.

16. Griffiths, E.J., and A.P. Halestrup. 1993. Protection by cyclopenthia A of ischemic reperfusion-induced damage in isolated rat hearts. J. Mol. Cell. Cardiol. 25:1461–1469.

17. Trost, L.C., and J.J. Lemasters. 1996. The mitochondrial permeability transition: a new pathophysiological mechanism for R eye’s syndrome and toxic liver injury. J. Pharmaol. Exp. Ther. 278:1000–1005.

18. Schinder, A.F., E.C. Olson, N.C. Spitzer, and M. Montal. 1996. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J. Neurosci. 16:6125–6133.

19. Ichas, F., L.S. Jouavil, and J.-P. Mazae. 1997. Mitochondria are excitable organelles capable of generating and conveying electric and calcium currents. Cell. 89:1145–1153.

20. Nicoll, A., E. Bassó, V. Petronilli, R.M. Weng, and P. Bernardi. 1996. Interactions of cyclophilin with mitochondrial inner membrane and regulation of the permeability transition pore, a cyclophilin A-sensitive channel. J. Biol. Chem. 271:2185–2192.

21. Brustovetsky, N., and M. Klingenberg. 1996. Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca2+. Biochemistry. 35:8483–8488.

22. Beutner, G., A. Rück, B. Riede, W. Welte, and D. Brdiczka. 1996. Complexes between kinases, mitochondrial porin, and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett. 396:189–195.

23. Halestrup, A.P., K.-Y. Woodfield, and C.P. Connern. 1997. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocator. J. Biol. Chem. 272:3346–3354.

24. White, R.J., and I.J. Reynolds. 1996. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. J. Neurosci. 16:5688–5697.

25. Pastorino, J.G., G. Simbula, K. Yamamoto, P.A.J. Glasscott, R.J. Rothman, and J.L. Farber. 1996. The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. J. Biol. Chem. 271:29792–29799.

26. Susin, S.A., N. Zamzami, M. Casteño, E. Daugas, H.-G. Wang, S. Geley, F. Fassy, J. Reed, and G. Kroemer. 1997. The central executioner of apoptosis. Multiple links between protease activation and mitochondria in Fas/Apo-1/CD95- and ceramide-induced apoptosis. J. Exp. Med. 186:25–37.

27. Hortelano, S., B. Dallaporta, N. Zamzami, T. Hirsch, S.A. Susin, I. Marzo, L. Bosca, and G. Kroemer. 1997. Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. FEBS Lett. 410:373–377.

28. Shiono, S., Y. Eguchi, W. Kamiike, S. Wager, U. Uchiyama, H. Matsuda, and Y. Tsujimoto. 1996. Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. J. Bioenerg. Biomembr. 28:129–136.

29. Decaudin, D., S. Geley, T. Hirsch, M. Casteño, P. Marchetti, A. Macho, R. Koffer, and G. Kroemer. 1997. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res. 57:62–67.

30. Boise, L.H., and C.B. Thompson. 1997. Bcl-XL can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc. Natl. Acad. Sci. USA. 94:3759–3764.

31. Kim, C.N., X.D. Wang, Y. Huang, A.M. Ibrado, L. Liu, G.F. Fang, and K. Bhalla. 1997. Overexpression of Bcl-x(L), inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cardiovasc. Res. 37:3115–3120.

32. Xiang, J., D.T. Chao, and S.J. Korsmeyer. 1996. Bax-induced cell death may not require interleukin 1beta-converting enzyme-like proteases. Proc. Natl. Acad. Sci. USA. 93:14559–14563.

33. Minn, A.J., P. Vélez, S.L. Schendel, H. Liang, S.W. Muchmore, S.W. Fesik, M. Fill, and C.B. Thompson. 1997. Bcl-XL forms an ion channel in synthetic lipid membranes. Nature. 385:353–357.

34. Schendel, S., Z. Xie, M.O. Montal, S. Matsuya, M. Montal, and J.C. Reed. 1997. Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. USA. 94:5113–5118.

35. Antonsson, B., F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Martinou, M. Bernasconi, A. Bernard, J.-J. Mermod, G. Mazzoli, et al. 1997. Inhibition of Bax channel-forming activity by Bcl-2. Science. 277:370–376.

36. Mittl, P.R.E., S. Dimarco, J.F. Krebs, X. Bái, D.S. Karanewsky, J.P. Priestle, K.J. Tomaselli, and M.G. Grutter. 1997. Structure of recombinant human CPP32 in complex with the tetrapeptide Acetyl-Asp-Val-Ala-Asp fluoromethyl ketone. J. Biol. Chem. 272:6539–6547.

37. Fernandes-Alnemri, T., R.C. Armstrong, J. Krebs, S.M. Srinivasula, L. Wang, F. Bullrich, L.C. Friz, J.A. Trapani, K.J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human ap-
optotic cysteine proteases containing two FADD-like domains. Proc. Natl. Acad. Sci. USA. 93:7464–7469.
38. Folch, J., M. Lees, and G.M.S. Stanley. 1957. A simple method for the isolation and purifi-
cation of total lipids from animal tissues. J. Biol. Chem. 226:447–506.
39. Colas des Francs-Small, C., F. Amand-Bretteville, A. Dar-
pas, M. Sallantin, J.-C. Huet, J.-C. Pernollet, and R. Rémy. 1992. Variation of the polypeptide composi-
tion of mitochondria isolated from different potato tissues. Plant Physiol. (Lond.) 98:273–278.
40. Knoll, H.R., W.F. Taylor, and W.W. Wells. 1973. Effects of energy metabolism on in vivo distribution of hexokinase in brain. J. Biol. Chem. 248:5414–5417.
41. Aroha, K.K., D.M. Parry, and P.L. Pedersen. 1992. Hexoki-
nase receptors: preferential enzyme binding in normal cells to nonmitochondrial sites and in transformed cells to mitochondrial sites. J. Bioenerg. Biomembr. 24:47–53.
42. Gelb, B., V. Adams, S. Jones, L. Griffin, G. MacGregor, and E. Mccabe. 1992. Targeting of hexokinase 1 to liver and hepato-
toma mitochondria. Proc Natl. Acad. Sci. USA. 89:202–206.
43. O’Gorman, E., G. Beutner, M. Dolder, A.P. Koretsky, D.
Brédyczka, and T. W allmann. 1997. The role of creatine ki-
node in inhibition of mitochondrial permeability transition. FEBS Lett. 414:253–257.
44. N leminen, A.L., A.M. Byrne, B. Herman, and J.I. Lemasters. 1997. Mitochondrial permeability transition induced by t-BuOOH: NAD(P)H and reactive oxygen species. Am. J. Physiol. 41:C1286–C1294.
45. R igaud, J.L., M.T. Paternostre, and A. Bluzat. 1988. Mechan-
isms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 2. Incorporation of the light-driven proton pump bacteriorhodop-
sin. Biochemistry. 27:2677–2686.
46. N ew, R.R.C. 1990. Preparation of liposomes. In Liposomes: a Practical Approach. R.R.C. New, editor. Oxford University Press, Oxford. 33–104.
47. M urphy, A.N., D.E. Bredesen, G. Cortopassi, E. Wang, and G. Fiskum. 1996. Bcl-2 potentiates the maximal calcium up-
take capacity of neural cell mitochondria. Proc. Natl. Acad. Sci. USA. 93:9893–9898.
48. Y ingl, X.M., Z.N. Oltvai, and S.J. Korsmeyer. 1994. BH 1 and BH 2 domains of Bcl-2 are required for inhibition of apo-
tosis and heterodimerization with Bax. Nature. 369:321–323.
49. E nari, M., R.V. Talanian, W.W. Wong, and S. Na-gata. 1996. Sequential activation of ICE-like and CPP32-like prote-
ases during Fas-mediated apoptosis. Nature. 380:723–726.
50. Kuida, K., T.S. Zheng, S. Na, C.-Y. Kyan, D. Yang, H. Karasuyama, P. R akic, and R.A. Flavell. 1996. Decreased apo-
tosis in the brain and premature lethality in CPP32-defi-
cient mice. Nature. 384:368–372.
51. Fraser, A., and G. Evan. 1996. A license to kill. Cell. 85: 781–784.
52. Kharbanda, S., P. Pandey, L. Schofield, S. Israels, R. Roncin-
ske, K. Y os hid a, A. Bharti, Z.-M. Yan, S. Saxena, R. W eich-
selbaum, et al. 1997. Role for Bcl-XL as an inhibitor of cyto-
solic cytochrome c accumulation in DNA damage-induced apoptosis. Proc. Natl. Acad. Sci. USA. 94:6939–6942.
53. vander Heiden, M.G., N.S. Chandal, E.K. Williamson, P.T. Schumacker, and C.B. Thompson. 1997. Bcl-XL regulates the membrane potential and volume homeo-stasis of mitochondria. Cell. 91:627–637.
54. Zhou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to C. elegans Ced-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 90:405–413.
55. Strasser, A., A.W. Harris, D.C.S. Huang, P.H. Krammer, and S. Cory. 1995. Bcl-2 and Fas APO-1 regulate distinct path-
ways to lymphocyte apoptosis. EMBO (Eur. Mol. Biol. Or-
gan.) J. 14:6136–6147.
56. Chiu, V.K., C.M. Walsh, L. Chau-Ching, J.C. Reed, and W.R. Clark. 1995. Bcl-2 blocks degradation but not Fas-
based cell-mediated cytotoxicity. J. Immunol. 154:2023–2029.
57. Memon, S.A., M.B. Moreno, D. Petrack, and C.M. Zachar-
chuk. 1995. Bcl-2 blocks glucocorticoid- but not Fas- or ac-
tivation-induced apoptosis in a T cell hybridoma. J. Immunol. 155:4644–4652.
58. Huang, D.C.S., S. Cory, and A. Strasser. 1997. Bcl-2, Bcl-
XL, and adenosine protein E1B19KD are functionally equival-
ent in their ability to inhibit cell death. Oxygen. 14:405–414.
59. Miura, M., C.B. Thompson. 1997. Apaf-1, a human protein homologous to the C. elegans death gene ced-3. Cell. 90:653–660.
60. Lacroix, V., A. Mignon, M. Fabre, B. Viollet, N. Rou-
quet, T. Molina, A. Porteu, A. Henrion, D. Bouscary, P. Varlet, et al. 1996. Bcl-2 protects from lethal hepatic apop-
tosis induced by an anti-Fas antibody in mice. Nat. Med. 2:80–86.
61. Cheng, E.H.Y., D.G. Kirsch, R.J. Clem, R. Avi, M.B. Ka-
tani, A. Bedi, K. Ueno, and J.M. Hardwick. 1997. Con-
version of Bcl-2 to a Bax-like death effector by caspases. Sci-
e. 278:1966–1968.
62. Uchum, S.W., M. Sattler, H. Liang, R.P. Meadows, J.E. Harlan, H.S. Yoon, D. Nettel, S.B. Chang, C.B. Thompson, S.-L. Wong, et al. 1996. X-ray and NMR structure of human Bcl-XL, an inhibitor of programmed cell death. Nature. 381:335–341.
63. Martin, S.J., and D.R. Green. 1995. Protease activation dur-
ing apoptosis: death by a thousand cuts? Cell. 82:349–352.