The Pro-apoptotic Proteins, Bid and Bax, Cause a Limited Permeabilization of the Mitochondrial Outer Membrane that Is Enhanced by Cytosol

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Abstract. During apoptosis, an important pathway leading to caspase activation involves the release of cytochrome c from the intermembrane space of mitochondria. Using a cell-free system based on Xenopus egg extracts, we examined changes in the outer mitochondrial membrane accompanying cytochrome c efflux. The pro-apoptotic proteins, Bid and Bax, as well as factors present in Xenopus egg cytosol, each induced cytochrome c release when incubated with isolated mitochondria. These factors caused a permeabilization of the outer membrane that allowed the corelease of multiple intermembrane space proteins: cytochrome c, adenylate kinase and sulfite oxidase. The efflux process is thus nonspecific. None of the cytochrome c-releasing factors caused detectable mitochondrial swelling, arguing that matrix swelling is not required for outer membrane permeability in this system. Bid and Bax caused complete release of cytochrome c but only a limited permeabilization of the outer membrane, as measured by the accessibility of inner membrane-associated respiratory complexes III and IV to exogenously added cytochrome c. However, outer membrane permeability was strikingly increased by a macromolecular cytosolic factor, termed PEF (permeability enhancing factor). We hypothesize that PEF activity could help determine whether cells can recover from mitochondrial cytochrome c release.

Key words: apoptosis • Bid • Bax • cytochrome c • mitochondrial outer membrane

The translocation of cytochrome c from the mitochondrial intermembrane space to the cytoplasm plays a crucial role in apoptosis (reviewed by Reed, 1997). In cell-free systems derived from mammalian somatic cells (Li et al., 1996) or Xenopus eggs (Kluck et al., 1997a,b), and in whole cells (Li et al., 1996; Kluck et al., 1997a; Yang et al., 1997; Bossy-Wetzel et al., 1998), cytochrome c is released from mitochondria during apoptosis. Once in the cytoplasm, cytochrome c switches on the cell-dismembering caspases by complexing with Apaf-1, procaspase-9, and dATP (Li et al., 1997b). In some cell types the cytochrome c molecule can be both necessary and sufficient for the activation of an apoptotic pathway, as the introduction of cytochrome c into cells by microinjection (Li et al., 1997a; Zhivotovsky et al., 1998) is capable of initiating apoptosis, and microinjection of cytochrome c neutralizing antibody abrogates apoptosis (Neame et al., 1998; Juin et al., 1999). Furthermore, in knockout mice lacking either caspase-9 (Hakem et al., 1998; Kuida et al., 1998) or Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998), reduced apoptosis in the brain can be linked to the absence of a program normally activated by cytosolic cytochrome c.

A major unanswered question concerns how cytochrome c leaves mitochondria during apoptosis. One potential mechanism involves mitochondrial swelling, either due to opening of the permeability transition pore in the inner membrane (Bernardi, 1996; Susin et al., 1997, 1999b) or to mitochondrial hyperpolarization (Vander Heiden et al., 1999, 1997). However, it is questionable whether such

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mitochondrial swelling occurs in most instances of apoptosis or is required for cytochrome c release. Electron micrographs of apoptotic cells frequently contain apparently intact unwollen mitochondria (Searle et al., 1975; Mancini et al., 1997; Zhuang et al., 1998; Martinou et al., 1999). Further, it has been reported that the pro-apoptotic proteins Bid (Luo et al., 1998) and Bax (Eskes et al., 1998; Jurgenmeier et al., 1998; Finucane et al., 1999) can release cytochrome c from isolated mitochondria in the absence of detectable mitochondrial swelling.

Exactly how Bid, Bax, and related proteins function to cause cytochrome c release is unclear. In cells given certain apoptotic stimuli, Bid or Bax can translocate to mitochondria (Wolter et al., 1997; Luo et al., 1998; Desagher et al., 1999) to initiate the release of cytochrome c (Jurgenmeier et al., 1998; Li et al., 1998; Luo et al., 1998; Narita et al., 1998; Rosse et al., 1998). In some cases, translocation of these proteins may require changes in their conformation (Desagher et al., 1999; Nechushtan et al., 1999). The formation of ion channels in synthetic lipid bilayers by some members of the Bcl-2 family such as Bax (Antonsson et al., 1997; Schlesinger et al., 1997), Bcl-xL (Minn et al., 1998), and Bcl-2 (Schendel et al., 1997) suggests that pro-apoptotic members of this family might interact directly with the outer mitochondrial membrane to allow efflux of cytochrome c. Ion channel formation by anti-apoptotic members of the Bcl-2 family has been proposed to prevent hyperpolarization (Vander Heiden et al., 1997) and thus indirectly counteract closure of the voltage-dependent anion channel (VDAC)1 on the outer membrane (Vander Heiden et al., 1999), although other studies report that the outer membrane is freely permeable to small ions (Mannella, 1992). Alternatively, Bax has recently been reported to interact directly with VDAC on the outer membrane to release cytochrome c (Narita et al., 1998; Shimizu et al., 1999) or with ANT on the inner membrane to initiate the permeability transition, indirectly leading to cytochrome c release (Marzo et al., 1998). More recently, intriguing studies report that Bax may cause instability in artificial lipid membranes, suggesting another mechanism by which Bax may permeabilize the outer mitochondrial membrane (Basanez et al., 1999). An important caveat in this regard is that Bcl-xL did not prevent these effects, whereas it is known to block cytochrome c release from intact mitochondria. The three-dimensional structures of Bid (Chou et al., 1999; MCDonnell et al., 1999) and Bcl-xL (Muchmore et al., 1996; Sattler et al., 1997) are similar, suggesting that Bid may also form ion channels. However, studies from Desagher et al. (1999) have suggested that Bid requires the cooperation of relatives such as Bax for its effect.

Here we examine the mitochondrial changes associated with apoptotic cytochrome c efflux in the cytosol-based Xenopus egg cell-free system. Bid and Bax, as well as pro-apoptotic factors endogenous to the Xenopus system, initiated the release of multiple intermembrane space proteins, namely cytochrome c, adenylate kinase (AK) and sulfite oxidase (SOx). Despite these alterations in outer membrane permeability, both the size and ultrastructural morphology of mitochondria remained unchanged, results inconsistent with a mechanism for release of cytochrome c requiring swelling of the mitochondrial matrix and rupture of the outer membrane.

Interestingly, we found that Bid and Bax by themselves produced only a small permeabilization of the outer mitochondrial membrane. However, a much greater permeabilization occurred when mitochondria were incubated with Bid or Bax in cytosol. This secondary permeability change is the result of a novel cytosolic activity, termed permeability enhancing factor (PEF). We propose that the presence or regulation of PEF activity may help decide whether mitochondria can recover from the activation of an apoptotic program.

Materials and Methods

Materials

Horse heart cytochrome c was obtained from Sigma Chemical Co. or from A mersham. SOx was purified from rat and chicken and anti-rat SOx antibodies raised in rabbits as previously described (Barber and Neame, 1990; Neame et al., 1998). ACD-EDV-CHO and 2 va A-D-fmk were purchased from Biomol and dissolved in DMSO.

Xenopus Egg Extracts and Incubations

Xenopus egg extracts were prepared as described (Newmeyer, 1998; Newmeyer et al., 1994). The final egg cytosolic extracts were approximately two parts cytoplasm and one part buffer A (250 mM sucrose, 20 mM Hepes/KOH, pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 5 μg/ml cytochalasin B, and 50 μg/ml cycloheximide). Incubations were comprised of either crude egg extract that contained mitochondria, or of isolated Xenopus egg mitochondria (1 mg/ml as protein) incubated in either buffer B or Xenopus egg cytosol. A II cell-free incubations, including those with buffer only, were supplemented with an ATP regenerating system (10 mM phosphocreatine, 2 mM ATP, and 150 μg/ml creatine phosphokinase).

The buffer used in incubations was buffer A supplemented to 100 mM KCl (buffer B), as we found that 80 mM KCl was required for complete dissociation of cytochrome c (but not AK) from mitochondrial membranes after hypotonic lysis or exposure to either Bid or Bax (not shown; Margoliash and Bossard, 1983; Aikowan et al., 1991; Eskes et al., 1998).

Measurement of DEVDase Activity

To measure caspase-3-like activation, extract aliquots (2 μl) were incubated with DEVD-pNA (N-acetyl-A-a-Glu-Val-Ala-asp nitrilotriamide, 40 μM; Biomol) in 200 μl of a buffer (250 mM sucrose, 20 mM Hepes/KOH pH 7.5, 50 mM KCl, 2.5 mM MgCl2, and 1 mM DTT) similar to that used to make the egg extracts. Incubations were kept at 22°C and A450 development monitored over 30 min (SPECTRAmax 250 microplate spectrophotometer).

Isolation and Incubation of Rat Liver Mitochondria

Male Sprague Dawley rats (300–500 gm body weight) were killed with carbon dioxide and their livers were removed and placed in ice-cold sucrose solution (300 mM sucrose, 10 mM Tris-HCl pH 7.4, and 1 mM EDTA) for transport. A II subsequent procedures were performed at 0 or 4°C. Tissue was placed in ice-cold buffer C (60 mM sucrose, 210 mM mannitol, 10 mM KCl, 0.5 mM DTT; 10 mM succinate, 10 mM Hepes/KOH pH 7.5, 5 mM EGTA, 1 mM PMSF, 0.1% BSA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), diced, and aliquots transferred to fresh buffer in a 10-ml Wheaton Potter-Evelhjem tissue grinder. Using a drill-driven Teflon-coated pestle, aliquots were homogenized by 3–5 strokes. Total homogenate was diluted to 100 ml with buffer C and centrifuged twice at 2,000 g for 5 min to remove unbroken cells. Mitochondria were then pelleted from the supernatant by 10 min at 8,500 g (Sorvall RC5C), and washed twice before a final

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1. Abbreviations used in this paper: AK, adenylate kinase; FEISEM, field emission in-lens scanning electron microscopy; PEF, permeability enhancing factor; SOx, sulfite oxidase; tBid, truncated Bid; VDAC, voltage-dependent anion channel.
wash in buffer C without PM SF or BSA, then resuspension in a minimal volume. Protein concentration was estimated by the Buret method.

**Light Scatter**

Mitochondrial swelling was assessed by measuring the decrease in light scattering at 520 nm (see Fig. 1). Light scatter measurements of reconstituted extracts needed to be performed on aliquots diluted with buffer A (which already constitutes 1/3 of the extract), as A 530 of undiluted extract was in the nonlinear range (~3). A aliquots (40 μl) of reconstituted extracts were diluted with 260 μl buffer B and added to a 1-ml cuvette for measurement of A 530 in a Hitachi 2000 spectrophotometer. A positive control, mitochondria were swollen by incubation with the membrane disrupting peptide mastoparan from wasp venom (Sigma Chemical Co.), sometimes used as an inducer of permeability transition (Pfeiffer et al., 1995).

**Transmission Electron Microscopy**

Samples of extract containing mitochondria (5 μl) were placed in an Eppendorf tube and fixative (5 μl of 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) layered on top, so that after a few minutes on ice the pellets formed a congealed plug. Afer full fixation (2 h), samples were washed in cacodylate buffer, followed by secondary fixation in 4% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4. Samples were then washed in double distilled water and stained en bloc with 2% aqueous uranyl acetate overnight. Dehydration was carried out stepwise in 30, 50, 70, 90, 95, and 100% acetone/water solutions before embedding in Durcopan (E.M.S.). Thin sections (~100 nm thick) were cut and imaged with transmission electron microscopy on either a JEOL 100CX or Philips 410 microscope. Cross-sectional area measurements were performed using NIH Image program (1.61) and presented as mean ± SD, with statistical significance determined using the paired Student’s test.

**Field Emission In-Lens Scanning Electron Microscopy**

High resolution scanning electron microscopy was performed using the recent technology afforded by high-brightness sources used in in-lens field emission instruments (FEISEM), which provide resolution to 1 nm (Ailen et al., 1996). Reconstituted extracts were incubated at 22 °C with BaxxTAM or alamethicin (2 μg/ml; Sigma Chemical Co.) to induce cytochrome c release (confirmed by DEVDase activation and Western blot). A aliquots (10 μl) were mixed with buffer B (400 μl) to wash the mitochondria, and then placed on 5-mm-square silicon chips in chambers constructed from Eppendorf tubes. Mitochondria were spun onto the chips (5 min, 14,000 g), followed by removal of supernatant, and fixation in 100 μl 150 mM sucrose, 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl 2, 2% paraformaldehyde, 0.25% glutaraldehyde on ice, followed by post-fixation in OOSO 4 in 0.2 M cacodylate buffer, pH 7.4. A after 20 min in 1% aqueous uranyl acetate, the specimens were dehydrated through an ethanolt series, and critical point dried from CO 2 using A rkon (Freon121; IC), as intermediate solvent. The chips were then sputter coated with 4 nm chromium, and examined in a Topcon ISI DS 130F field emission, in-lens SEM at 30 kV accelerating voltage, with digital image acquisition.

**Western Blotting**

To determine mitochondrial and cytosolic content of various proteins, incubation aliquots were centrifuged (12,000 g) to pellet mitochondria and the supernatant carefully removed. For SDS-PAGE, supernatant samples (10 μl) were mixed with buffer B (400 μl) to wash the mitochondria, and then placed on 5-mm-square silicon chips in chambers constructed from Eppendorf tubes. Mitochondria were spun onto the chips (5 min, 14,000 g), followed by removal of supernatant, and fixation in 100 μl 150 mM sucrose, 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl 2, 2% paraformaldehyde, 0.25% glutaraldehyde on ice, followed by post-fixation in OOSO 4 in 0.2 M cacodylate buffer, pH 7.4. A after 20 min in 1% aqueous uranyl acetate, the specimens were dehydrated through an ethanolt series, and critical point dried from CO 2 using A rkon (Freon121; IC), as intermediate solvent. The chips were then sputter coated with 4 nm chromium, and examined in a Topcon ISI DS 130F field emission, in-lens SEM at 30 kV accelerating voltage, with digital image acquisition.

**Adenylate Kinase Activity**

M itochonndrial AK activity was measured by a modification of the method of Schmidt et al. (1984). Extract aliquots (50 μl) containing ~0.05 mg mitochondrial protein were pelleted at 12,000 g for 3 min, and the pellet washed twice in 800 μl buffer D (80 mM sucrose, 210 mM mannitol, 10 mM KCl, 0.5 mM DTT, 10 mM succinate, 10 mM Hepes/KOH, pH 7.5, and 5 mM EGTA) to remove contamination of cytosolic AKs. The mitochondria were lysed with 50 μl of 1% Triton X-100 in buffer D to release remaining AK and the sample stored at –80 °C. AK activity was measured in a mixture composed of 1 ml of 130 mM KCl, 6 mM MgSO 4, 100 mM Tris-HCl, pH 7.5, 15 μl of 0.1 M Na D H, and 5 μl each of 0.1 M ATP, 100 mM phosphoenolpyruvate, 1 mM rotenone, 1.5 mM oligomycin, a mixture of pyruvate kinase and lactate dehydrogenase (80 U/ml each), and 0.15 M A M P. 200 μl of buffer mix was added to 6 μl of sample in a microtiter plate. The absorbance decrease of NADH was measured at 366 nm in a microtiter plate reader (SPECTRAmax R 250) for 10 min at 22 °C. The rates were calculated (SOF T MAX PR O) and calibrated against chicken muscle myokinase (Sigma) activity. Rates obtained in the presence of the inhibitor di-adenosine pentaphosphate (400 μM; Sigma) were minimal and subtracted as background.

**Complex IV Accessibility (Cytochrome c Oxidase Latency Assay)**

To examine the intactness of the outer mitochondrial membrane we measured the accessibility of cytochrome c oxidase to exogenous cytochrome c. The assay (modified from Wojtczak et al., 1972) involved the addition of extract (2 μl) containing mitochondria (final protein concentration ~9 μg/ml) to 450 μl assay solution in a 1-ml cuvette. The assay solution consisted of 100 μM horse heart cytochrome c, 60 mM KCl, 125 mM sucrose, 20 mM Tris-HCl, pH 7.4, 1 μM each of carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), rotenone and antimycin, and sufficient sodium dithionite crystals to obtain near fully reduced cytochrome c (A 550/A 565 between 6 and 10). On addition of mitochondria, samples were quickly mixed and A 550 recorded every 6 s (H I tachi 2000). The rate of cytochrome c oxidation at 550 nm integrated over 30 s reflected complex IV accessibility to the solution. M animal complex IV accessibility was obtained by pre-mixing 2 μl extract with 2 μl Triton X-100 (2%) and used to calculate percent complex IV accessibility.

**Complex III Accessibility (Quinol–Cytochrome c Assay)**

A n additional method used to assess permeability of the mitochondrial outer membrane was the reduction of exogenous cytochrome c by complex III (cytochrome bc 2). Degli Esposti et al., 1982. M itochondria from treated extracts (250 μl) were washed by dilution in 1.25 ml buffer E (125 mM sucrose, 60 mM KCl, 20 mM Tris-HCl, pH 7.4), followed by pelleting for 5 min at 12,000 g. The mitochondrial pellet was resuspended in 250 μl buffer E and 25 μl (final concentration 0.15 mg/ml) added to 300 μl buffer E containing 2 mM potassium cyanide and (to block oxidation by complex IV). Decyl benzoxquinol (DBH 2, 5 μl of 3.5 mM stock in ethanol) was added and the sample sealed with parafilm and mixed. Finally, 10 μl of 2.5 mM ferricytochrome c (horse heart; Sigma Chemical Co.) was added with A H amilton syringe and the rate of cytochrome c reduction at 550 nm was integrated over 30 s.

**Production of Recombinant Bcl-2 Family Proteins**

Bcl-2 and the control β-galactosidase were expressed in baculovirus-infected SF-9 cells, and lysates were prepared as described (Neumeyer et al., 1994). Human BID (hBID) cDNA was amplified with PCR and sub-cloned into the BamHI and NotI sites in pGEX4T-1 (Pharmacia). BL21(DE3) cells were transformed and GST-hBID induced with 1 mM isoprropyl β-thiogalactopyranoside (IPTG) at 37 °C for 4 h. The induced protein was incubated with glutathione sepharose (Pharmacia) and eluted with 20 mM reduced glutathione. Euluted GST-hBID was digested with thrombin (Pharmacia) to cleave GST, which was subsequently removed by incubation with a new batch of glutathione sepharose. The HbID product was ~90% pure with minor contamination of GST. Human caspase-8 lacking the prodomain in PET15b vector (Novagen) was expressed in BL21(DE3) cells by induction with 1 mM IPTG at 37 °C for 4 h and affinity-purified with Ni 2+ -NTA (Q Iagen). The protein was eluted with 50 mM and 250 mM imidazole, and the 250 mM imidazole eluate further purified on a 1 ml Q-Sepharose FF HiTrap column (Pharmacia) using a 0-0.6 M KCl linear gradient. The product was fully processed and electrophoretically
Characterization of Cytosolic PEF

Cytosol was size-fractionated using Millipore Ultrafree-MC centrifugal ultrafiltration membranes. Cytosol was dialyzed against 4 changes of buffer B (500 ml each) over 24 h at 4°C in 3500 MW cutoff dialysis tubing (Spectra/Por). Cytosolic proteins were hydrolyzed by incubation with 0.1 mg/ml proteinase K or 1.0 mg/ml trypsin (Sigma Chemical Co.) for 30 min at 22°C, followed by addition of phenylmethylsulfonyl fluoride (1 mM). Heat-denatured cytosol was prepared by heating at 50°C for 15 min or 95°C for 1 min, followed by clearing at 12,000 g for 5 min.

Results

Absence of Gross Mitochondrial Swelling Accompanying Cytochrome c Release from Xenopus Mitochondria

To determine if mitochondrial swelling might participate in the efflux of mitochondrial cytochrome c during apoptosis in the Xenopus cell-free system, we assessed mitochondrial swelling by three methods. First, we measured the light scatter of mitochondrial suspensions. As mitochondria swell, their refractive index decreases, resulting in a drop in spectrophotometric absorbance at higher wavelengths (Knight et al., 1981). X. laevis mitochondria were induced to release cytochrome c by incubation in Xenopus cytosol for 4 h at 22°C. We found that these mitochondria did not exhibit decreased light scattering (Fig. 1) despite complete cytochrome c release. As a control to determine if mitochondrial swelling could be measured with Xenopus mitochondria using this technique, we used mastoparan, a membrane disrupting peptide that causes mitochondrial swelling associated with permeability transition (Nicolaus et al., 1994; Pfeiffer et al., 1995; Fig. 2) and cytochrome c release (Ellerby et al., 1997). As shown in Fig. 1, mastoparan at 300 and 400 µg/ml rapidly induced both a decrease in light scattering and loss of mitochondrial cytochrome c. We conclude that the constitutive pro-apoptotic factor present in the Xenopus cell-free system initiates cytochrome c release by a mechanism independent of mitochondrial swelling, as measured by light scatter.

Second, we used transmission electron microscopy to compare the size and morphology of apoptotic and non-apoptotic mitochondria (Fig. 2, A and B). Crude egg extracts were incubated at 22°C to induce mitochondrial cytochrome c loss. In the Xenopus system, essentially all mitochondrial cytochrome c becomes cytosolic ~30 min before the detection of DEVDase activation (Kluck et al., 1997a,b). Samples fixed immediately after full DEVDase activation exhibited no obvious mitochondrial swelling or morphological changes when compared with (non-apoptotic) extracts that had been incubated on ice. Quantitative assessment of mitochondrial cross-sectional area indicated no difference (P = 0.32, n = 38) between apoptotic (0.75 ± 0.25, arbitrary units) and non-apoptotic mitochondria (0.77 ± 0.26). The integrity of the outer membrane appeared equal in apoptotic and non-apoptotic samples. Comparison of several individual mitochondria by three-dimensional electron tomography (Renken, C., and G. Perkins, unpublished data) also failed to reveal obvious changes in outer membrane ultrastructure. Mitochondria fixed at 3 h after cytochrome c release also were unswollen (not shown), indicating that neither activated caspases nor other cytosolic factors cause gross morphological mitochondrial changes.

The majority of mitochondria in Xenopus extracts was observed to be in the condensed form (dark matrix and wide cristae), rather than the orthodox configuration (light matrix and narrower cristae). A condensed matrix is thought to reflect low respiration (Hackerbrock, 1968; Ord and Smith, 1982). Thus, the condensed morphology of Xenopus extract mitochondria may indicate low respiratory rates in response to the high external energy status caused by addition of the ATP regenerating system (see Materials and Methods). Alternatively, unfertilized Xenopus eggs may contain a factor similar to that implicated in the condensed mitochondrial morphology of unfertilized sea urchin eggs (Innis et al., 1976). Condensed mitochondria have recently been observed in association with apoptosis (Mancini et al., 1997; Zhuang et al., 1998). With the opening of the permeability transition pore, mitochondria...
in the condensed state are converted irreversibly (in the presence of sucrose) to the orthodox conformation. Thus, simply on ultrastructural grounds, as both apoptotic and non-apoptotic *Xenopus* mitochondria are condensed, it is unlikely that a permeability transition had taken place.

To examine further the ultrastructure of apoptotic mitochondria, we used a third approach, a high-resolution form of scanning electron microscopy (FEISEM; Allen and Goldberg, 1993; Allen et al., 1996). As Fig. 2 shows, FEISEM revealed that mitochondria depleted of cytochrome *c* were of similar size and appearance to control mitochondria. Outer membrane discontinuities were not observed. This technique did reveal outer membrane pores, 10–13 nm in diameter, in mitochondria treated with the antibiotic peptide alamethicin (Fig. 2 F), which also induced complete release of cytochrome *c* (Kluck and Newmeyer, unpublished). However, no such pores were observed in mitochondria depleted of cytochrome *c* by the factors present in *Xenopus* cytosol, or by Bid or Bax, suggesting that if these factors form pores, they are either transient or considerably smaller than those formed by alamethicin (Woolley and Wallace, 1992). In summary, multiple approaches (Figs. 1 and 2) demonstrated the absence of gross ultrastructural changes in mitochondria accompanying cytochrome *c* release in the *Xenopus* cell-free system.

**Adenylate Kinase Loss from the Intermembrane Space Precedes Caspase Activation**

To continue investigating the mitochondrial events leading to cytochrome *c* release, we next determined whether other soluble proteins present in the intermembrane space were released during apoptosis. One such protein is adenylate kinase (25,200 Da). The amount of mitochondrial adenylate kinase (AK) released into the cytosol is difficult to measure because cytosol already contains ~50% of the cell’s AK activity (not shown). However, the AK activity remaining in the mitochondrial pellet can readily be assayed. We incubated mitochondria in cytosol for various times, washed them free of cytosol, and measured adenylate kinase content in the final pellet. As Fig. 3 shows, with apoptosis induced by *Xenopus* cytosol, almost all AK activity was lost from mitochondria before DEVDase activation, with kinetics similar to those observed for cytochrome *c*.

**Sulfite Oxidase Is Coreleased with Cytochrome c from Rat Liver Mitochondria**

Another mitochondrial protein, sulfite oxidase (SOx), is found only in the intermembrane space. Antisera raised to rat SOx failed to recognize the *Xenopus* protein, we determined whether rat liver mitochondria incubated in *Xenopus* extracts coreleased SOx with cytochrome *c*. Many of the apoptotic features of the *Xenopus* egg cell-free system were duplicated if rat liver mitochondria were incubated in *Xenopus* cytosol. That is, mitochondrial cytochrome *c* was released after a period of incubation at 22°C, but not at 0°C (Fig. 3). (It should be noted that, for reasons unknown, some preparations of rat liver mitochondria failed to exhibit cytochrome *c* release in response to incubation with Bid, Bax or *Xenopus* egg cytosol.) Near the time of rat cytochrome *c* release, *Xenopus* cytosolic
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DEVDases became activated, and coincubated rat liver nuclei exhibited chromatin condensation, margination and beading, with subsequent nuclear disintegration (not shown) typical of apoptosis in the *Xenopus* system. Swelling of the rat liver mitochondria was not observed by cross-sectional area measurements of transmission electron micrographs (P = 0.5, n = 21), with non-apoptotic mitochondria showing an average area of 2.45 ± 1.1 (arbitrary units), compared with 2.45 ± 1.0 for apoptotic mitochondria. Light scatter measurements (not shown) also demonstrated a lack of swelling. Despite this absence of swelling, we found that SOx (a dimer of 52 kD subunits) was coreleased from the intermembrane space with cytochrome c (Fig. 3) and with A K (not shown). Loss of these proteins from mitochondria occurred over a period of under 1 h.

As SOx is normally found only in the intermembrane space, it was of interest to examine whether the presence of this protein in cytosol altered the pro-apoptotic activity of cytochrome c. Purified rat or chicken SOx added to *Xenopus* cytosol, either in the presence or absence of cytochrome c, did not alter DEVDase activation (not shown), indicating that SOx does not affect caspase activation. (It is unlikely that released mitochondrial A K has any effect on the activation of caspases, as much of the cellular A K is already present in cytosol.) Western blot analysis of rat SOx released from mitochondria suggested that this protein was degraded in apoptotic cytosol, but not in either buffer (Fig. 3) or an ultrafiltrate obtained by passing cytosol through a 10-kD cutoff membrane (not shown). Chicken SOx was cleaved to a 45-kD fragment after a 1-h incubation with either caspase-3 or cytochrome c-activated cytosol, and this cleavage was blocked by the caspase inhibitor Ac-DEVD-CHO (1 μM, not shown). However, while chicken SOx appeared to be a good caspase substrate, rat SOx was cleaved in a much less efficient manner to several products of different molecular mass. In summary, the corelease of cytochrome c, A K and SOx with apoptosis, argues against an outer membrane transport channel specific for cytochrome c, and is consistent with the formation of a nonspecific opening or protein channel in the outer mitochondrial membrane.

**Complex IV Accessibility Changes Coincide with the Release of Cytochrome c and Are Prevented by Bcl-2**

To examine in more detail the apoptotic changes occurring in mitochondrial outer membrane permeability, we measured the accessibility of exogenous cytochrome c to complex IV (cytochrome c oxidase), present on the outside face of the inner mitochondrial membrane. When detergent-treated or mechanically disrupted mitochondria are suspended in a solution containing reduced cytochrome c, this exogenous cytochrome c becomes gradually oxidized by complex IV, leading to a steady change in the absorption spectrum of cytochrome c. In intact mitochondria, this change does not occur, because the permeability barrier of the outer membrane prevents the interaction of exogenous cytochrome c with the catalytic site exposed within the intermembrane space (Wikstrom and Casey, 1985). The rate of change of absorbance at 550 nm is taken as a measure of the accessibility of complex IV, and hence the permeability of the outer membrane. The values obtained were expressed as percentages of the rate of change of A<sub>550</sub> observed after exposure of mitochondria to 1% Triton X-100, as this treatment was assumed to cause complex IV to be completely accessible. Carefully isolated *Xenopus* egg and rat liver mitochondrial preparations exhibited <1% of this value, while osmotic lysis of the outer membrane gave complex IV accessibility values close to 100%.

We used this assay to examine the permeability changes occurring in mitochondria incubated in *Xenopus* cytosol. With the onset of apoptosis, complex IV accessibility increased at around the time of cytochrome c release (Fig. 4), indicating that outer membrane permeability to cytochrome c in apoptotic mitochondria (in the presence of cytosol) is bidirectional. The addition of Bcl-2 to the extracts blocked both the increase in complex IV accessibility and the release of endogenous cytochrome c.
Both Bid and Bax Induce Mitochondrial Cytochrome c Release without Gross Mitochondrial Swelling

Next, we examined the effects of two known pro-apoptotic human proteins, Bid and Bax. Addition of recombinant Bid or Bax to reconstituted *Xenopus* egg extracts (mitochondria mixed with cytosol) caused the rapid release of mitochondrial cytochrome c (Fig. 5) and the activation of DEVDases (not shown). Cytochrome c release was complete and highly synchronous. Recombinant Bax exhibited a 1,000-fold lower specific activity than recombinant Bid (not shown); this may be due to the absence of the Bax transmembrane domain or to undefined differences in the way these proteins are expressed in bacteria. Cleavage of full-length Bid by caspase-8 (see Materials and Methods) to give truncated Bid (tBid) consistently enhanced its potency (not shown), as reported by others (Li et al., 1998; Luo et al., 1998).

Transmission electron microscopy of mitochondria depleted of cytochrome c by treatment with Bax or caspase-activated Bid revealed no significant changes in mitochondrial size ($P = 0.44$, $n = 21$ and $P = 0.33$, $n = 21$ for comparisons of control mitochondria with Bid- and Bax-treated mitochondria, respectively). The mean cross-sectional areas of control, Bid- and Bax-treated mitochondria were $0.684 \pm 0.2$, $0.70 \pm 0.2$, and $0.71 \pm 0.2$, respectively (arbitrary units). Thus, Bid and Bax, like the factors present in *Xenopus* egg cytosol, induced cytochrome c release without gross changes in mitochondrial morphology.

Adenylate Kinase Is Coreleased with Cytochrome c after Treatment with Bid and Bax

As cytosol-treated mitochondria coreleased AK with cytochrome c (Fig. 3), we examined whether this was also the case for tBid- and Bax-treated mitochondria. Indeed, tBid and Bax both released AK with kinetics similar to the release of cytochrome c (Fig. 5). This release occurred in the presence of either buffer or cytosol. The constitutive presence of large amounts of AK activity in the cytosol prevented us from measuring the AK released from mitochondria incubated in cytosol; however, for mitochondria

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**Figure 4.** Outer mitochondrial membrane permeability to cytochrome c is bidirectional in the *Xenopus* cell-free system. *Xenopus* mitochondria were incubated in cytosol at 0 or 22°C for 4 h in the presence of Sf-9 cell lysates (2% vol/vol) containing either β-galactosidase or Bcl-2 expressed after baculovirus infection. At the times indicated, 2-μl aliquots were assayed for either DEVDase activity (top) or complex IV accessibility (bottom).

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**Figure 5.** In the absence of cytosol, Bid and Bax release cytochrome c and adenylate kinase, but do not develop high complex IV accessibility. *Xenopus* egg mitochondria were incubated in buffer B or cytosol alone, or with added tBid (170 ng/ml) or Bax (37 μg/ml) for 4 h at 22°C. At the indicated times, aliquots were measured for (A) mitochondrial cytochrome c content, (B) mitochondrial adenylate kinase activity, and (C) complex IV accessibility. Data are single measurements except those for adenylate kinase (mean and SD of triplicates), and are representative of two experiments. (D) PEF acts subsequent to tBid, and tBid effects on the outer membrane are sustained. *Xenopus* mitochondria were first incubated at 22°C in buffer B with or without tBid (170 ng/ml) for 2 h to allow tBid to cause complete cytochrome c release (not shown). Mitochondria were then washed in buffer B and resuspended in fresh cytosol, and aliquots were tested every 15 min for complex IV accessibility.
incubated in buffer, the amount of AK released into the supernatant of tBid and Bax-treated mitochondria accounted for that lost from the mitochondrial pellet (not shown), indicating that the protein was translocated, rather than inactivated. These data support the hypothesis that similar cytochrome c-releasing mechanisms are used by various cytochrome c–releasing proteins.

Cytosol Greatly Increases Complex IV Accessibility Induced by Bid and Bax

Cytochrome c release from Xenopus mitochondria incubated in Xenopus cytosol is associated with high accessibility of the inner membrane protein cytochrome c oxidase (complex IV) to exogenous cytochrome c (Fig. 4). This high accessibility is also seen in Xenopus mitochondria treated with tBid and Bax in the presence of Xenopus cytosol (Fig. 5 C). It was surprising to note, however, that mitochondria treated with tBid and Bax in buffer displayed an almost undetectable level of accessibility of complex IV to exogenous cytochrome c (Fig. 5 C), despite their complete loss of endogenous cytochrome c (Fig. 5 A). These results imply that a novel cytosolic activity, which we call Permeability Enhancing Factor, or PEF, is responsible for a striking increase in complex IV accessibility beyond that produced by factors such as tBid or Bax.

PEF Can Act Downstream of tBid

PEF might function either simultaneously with factors like tBid or subsequent to their action. To address this question, we did the following experiment. Mitochondria were first depleted of cytochrome c by an initial incubation with tBid in buffer, and then washed and resuspended in cytosol for a second incubation. As Fig. 5 D shows, complex IV accessibility rapidly increased during this second incubation in cytosol. This is consistent with data showing that tBid stays strongly associated with mitochondria after washing (Kuwana, O., T. von Ahsen, and D.D. Newmeyer, unpublished results), but further implies that the effect of tBid is sustained. Some samples were incubated in fresh buffer before reincubation with cytosol, in an attempt to encourage maximum repair of the tBid-induced permeability. However, this intermediate incubation had no effect (not shown). We conclude from this experiment that PEF can function subsequent to tBid-induced cytochrome c translocation. This experiment also demonstrates that PEF activity is not dependent on downstream effects of cytochrome c release, because the cytosol used in Fig. 5 D contained neither cytochrome c (nor any other soluble proteins from the mitochondrial intermembrane space) nor active caspases.

Cytosol Increases Complex III Accessibility

It could have been argued that the quantitative differences in accessibility of complex IV reflected some effect specific for that enzyme complex, rather than changes in the permeability of the outer membrane. To rule out this possibility, we measured the accessibility of another mitochondrial inner membrane component, complex III. This assay is similar in principle to the complex IV assay, except that cytochrome c reduction rather than oxidation is measured, and an electron donor (DBH₂) is added to ensure saturating substrate (see Materials and Methods). These experiments showed that cytosol-induced cytochrome c release was accompanied by an increase in the accessibility of both complexes III and IV (Fig. 6, lane 4), confirming the bidirectional permeability of the outer membrane under these conditions. Again, however, in the presence of buffer alone, tBid induced a nearly undetectable accessibility of complexes III and IV (Fig. 6, lane 2), which was greatly enhanced in the presence of cytosol (Fig. 6, lanes 6 and 7). The ability of cytosol to increase outer membrane permeability was not due to a feedback effect of cytochrome c–activated caspases, as Ac-DEVD-CHO (10 μM) did not prevent the development of high complex III and IV accessibility (not shown).

In summary, the enhancing effect of cytosol on accessibility of complexes III and IV (Figs. 4–6) is explained by the presence of a factor in Xenopus cytosol, which we have named permeability enhancing factor (PEF). PEF does not release cytochrome c by itself, but rather enhances outer membrane permeability after a pro-apoptotic factor has caused the release of endogenous cytochrome c.
tBid and PEF Do Not Permeabilize the Mitochondrial Inner Membrane

To address the possibility that PEF increases accessibility of complexes III and IV by grossly perturbing the integrity of mitochondria, we examined whether a soluble mitochondrial matrix protein, mtHsp70, is released after the actions of tBid and PEF. First, to show that mtHsp70 can be released if the inner mitochondrial membrane is permeabilized, we treated mitochondria with increasing concentrations of digitonin and followed the redistribution of mtHsp70 from the mitochondrial pellet fraction to the soluble supernatant. Fig. 7A shows that cytochrome c is released at a low concentration (0.1%) of digitonin, but that mtHsp70 becomes soluble at higher digitonin concentrations (0.2%). Only at still higher concentrations (0.6%) did the membrane-associated Rieske protein become solubilized, showing that at the intermediate concentrations sufficient to release mtHsp70, digitonin did not solubilize the mitochondrial membranes, but merely permeabilized them. Next, we examined the behavior of mtHsp70 in mitochondria induced to release cytochrome c by cytosol or by tBid (in the presence or absence of cytosol). As seen in Fig. 7B, mtHsp70 remained in the mitochondrial pellet fraction under all conditions, even in the presence of cytosol containing active PEF. We conclude that cytosolic factors and tBid can permeabilize the outer mitochondrial membrane while leaving the inner membrane intact.

PEF Characteristics

To begin characterizing PEF, we treated cytosol in various ways before incubating it with Xenopus mitochondria and tBid (Fig. 8). Treatment with proteinase K (Fig. 8), trypsin, or heat (50°C, 15 min, or 95°C, 1 min; not shown) greatly diminished or abolished cytosol's ability to increase complex IV accessibility, indicating that PEF is proteinaceous. PEF activity was not observed in the ultracentrifugate obtained by passing cytosol through a 300-kD cutoff membrane, and was not lost on dialysis, suggesting that PEF is macromolecular and possibly present in a large complex. The complex IV accessibility produced by PEF accumulates over a few hours to approach that seen in mitochondria treated with Triton X-100 (see Materials and Methods) and digitonin (not shown). PEF activity is reduced substantially on dilution of cytosol. A PEF-like activity may also be present in mammalian cells, as a lysate (40 mg/ml) of human Jurkat cells caused an increase in complex IV accessibility of isolated Xenopus mitochondria (not shown). This Jurkat cell PEF-like activity was observed in the presence or absence of tBid, suggesting that the Jurkat cell lysate contained endogenous cytochrome c-releasing factors.

PEF Is Destroyed by Caspases

To address the possibility that PEF is a substrate of caspases, we examined the effect of cytochrome c-activated cytosol on fresh Xenopus mitochondria. Fresh cytosol was first incubated for 3 h with a low level (0.5 μM) of cytochrome c to activate cytosolic caspases (DEVDases). Subsequent addition of mitochondria to this activated cytosol resulted in rapid loss of mitochondrial cytochrome c and AK (by 1 h, not shown). The factor responsible for

![Figure 7](image)

Figure 7. tBid and PEF preserve the integrity of the mitochondrial matrix compartment, as measured by retention of a soluble mitochondrial matrix protein, mtHsp70. (A) mtHsp70 is released when mitochondria are treated with intermediate concentrations of digitonin. Xenopus egg mitochondria were treated with increasing concentrations of digitonin to selectively permeabilize the outer, then the inner mitochondrial membranes. After two hours incubation, the contents of the pellet and supernatant fractions were analyzed by immunoblotting (left panel) with antibodies to cytochrome c, mtHsp70, and the inner membrane-associated Rieske protein (part of complex III). A loss, accessibility of complex IV was measured as described above (right panel). Note that mtHsp70 is released by digitonin at 0.2%, a concentration insufficient to solubilize the Rieske protein, but more than sufficient to release cytochrome c. (B) mtHsp70 is not released by tBid and PEF, despite full release of cytochrome c and full accessibility of complex IV. Xenopus mitochondria were treated with 170 ng/ml tBid, in either the presence or absence of cytosol, and either at 0 or 22°C, as indicated. DEVD-CHO (10 μM) was added to cytosol incubations to minimize PEF degradation and thus maximize its activity. Ater 3 h of incubation, the contents of the mitochondrial pellet were analyzed by immunoblotting with antibodies to cytochrome c, mtHsp70 and Rieske protein (left), and the accessibility of complex IV (right). The samples were taken well after full cytochrome c release from mitochondria, which occurred in this experiment at 1 h in the tBid-treated sample and at 2 h in cytosol at 22°C (not shown). Note that mtHsp70 is retained in the pellet fraction despite full cytochrome c release and increased complex IV accessibility (reflecting PEF activity). (The bands stained by anti-Rieske antibody in cytosol are nonmitochondrial cross-reacting proteins.)
this cytochrome c efflux is likely to be a caspase-activated Bid-like factor (Kuwana, 1998), rather than caspases themselves, as addition of caspase inhibitors just before the addition of mitochondria did not block cytochrome c release. Strikingly, however, cytochrome c–activated cytosol did not produce high complex IV accessibility, even though it caused the complete release of cytochrome c from mitochondria (Fig. 9). Thus, this cytochrome c–treated cytosol contained activated DEVDases, inactivated PEF, and activated Bid-like activity.

Further, we found that the addition of caspase inhibitors to cytosol substantially enhanced and prolonged PEF activity. Typically, in the presence of cytosol, complex IV accessibility increased over 1–2 h after cytochrome c release and then reached a plateau (Fig. 9). If caspase inhibitors were added, however, this plateau was surpassed and complex IV accessibility approached 100% of that seen with Triton-treated mitochondria (Fig. 9). In summary (Fig. 10), cytosolic PEF begins to act on mitochondria once they have lost their cytochrome c, and over 2–3 h, progressively allows a higher permeability of the outer membrane to exogenous cytochrome c. If caspases are activated by the released cytochrome c, PEF activity is inactivated after perhaps 1–2 h. Addressing whether this inactivation by caspases might be direct or indirect awaits the molecular identification of PEF.

Discussion

We have investigated how mitochondrial cytochrome c is released during apoptosis in a cell-free system based on Xenopus egg extracts (Kluck et al., 1997a,b; Kuwana et al., 1998). Four pro-apoptotic cytochrome c–releasing activities were examined: (a) the spontaneous cytochrome c–releasing activity of Xenopus cytosol, (b) the Bid-like activity of cytochrome c–activated Xenopus cytosol, (c) recombinant human Bid activated with caspase-8, and (d) recombinant Bax. These factors all caused a permeability of the outer membrane that allowed the corelease of AK (and SOx, where measurable) with cytochrome c. None of these cytochrome c–releasing factors caused detectable mitochondrial swelling (Figs. 1 and 2). From this and other data, we conclude that neither mitochondrial permeability transition nor hyperpolarization occur in this apoptotic system.

A potentially attractive mechanism for the release of multiple proteins from the intermembrane space would in-
Mitochondrial Permeabilization by Apoptotic Factors

Figure 10. Model of two types of mitochondrial outer membrane permeability observed in X enonpus extracts during apoptosis. Mitochondria treated with tBid or Bax in buffer undergo efflux of endogenous cytochrome c (12 pmol/μl mitochondria) across the outer membrane, with limited permeability to exogenous cytochrome c. A additional exposure to cytosol (PEF) allows high permeability to exogenous cytochrome c (60 nmol/μl mitochondria per min, as measured by the rate of the complex IV reaction). Thus, this increased exchange of endogenous cytochrome c is at least ~5,000 times the rate of cytochrome c efflux after tBid or Bax treatment, assuming that all of the mitochondrial cytochrome c is released over 1 min. PEF begins to act immediately after tBid-induced permeabilization, but is inactivated over 1-2 h by caspase activation. The inner membrane appears unaffected.

Involves swelling of the mitochondrial matrix, leading to mechanical disruption of the outer mitochondrial membrane. A single break in the outer mitochondrial membrane is theoretically sufficient to allow cytochrome c to diffuse rapidly out of mitochondria down its concentration gradient (10 mM in intermembrane space and cristae). Ruptures in the outer membrane ensue if the matrix swells past the point that the outer membrane can expand. Such matrix swelling is proposed to occur during apoptosis in response either to the formation of a 1.5-kD permeability transition pore in the inner membrane (Bernardi, 1996; Petit et al., 1996; Susin et al., 1997) or to hyperpolarization of the inner membrane (Vander Heiden et al., 1997, 1999).

However, mitochondrial swelling has not been observed as a frequent accompaniment to apoptosis. In the studies reported here, light scatter measurements (Fig. 1) and transmission and scanning electron microscopy (Fig. 2) all failed to provide evidence of mitochondrial swelling in association with apoptotic cytochrome c efflux from X enonpus mitochondria. A mother group has reported that, in whole cells of X enonpus embryos treated with gamma irradiation, apoptotic mitochondria display normal size and morphology (Hensey and Gautier, 1997). In other systems also, mitochondrial swelling has not been observed with apoptosis (Searle et al., 1975; Mancini et al., 1997; Zhuang et al., 1998), and in some apoptotic cells mitochondria even shrink (Martinou et al., 1999). Previously, Bid was reported to release cytochrome c from isolated mitochondria without swelling (Luo et al., 1998). Bax-mediated cytochrome c release has been associated in some reports with mitochondrial swelling (Narita et al., 1998) or permeability transition (Pastorino et al., 1998), but in other reports with an absence of swelling (Jurgensmeier et al., 1998; Martinou et al., 1999). The presence of swollen mitochondria in some apoptotic systems, including whole cells, may reflect either secondary necrosis or a fundamentally distinct apoptotic cytochrome c release mechanism.

Cytochrome c efflux is not a specific export process, as AK (25,200 D a) was also released by each of the four proapoptotic factors we studied. Furthermore, SOX (104 kD) was released from rat liver mitochondria incubated in cytosol (Fig. 3). Several intermembrane space proteins, including AK (Kohler et al., 1999; Samali et al., 1999; Single et al., 1998), a cytochrome c-GFP fusion protein (Hesk kanen et al., 1999), AIF (Susin et al., 1997, 1999b), and certain caspases (Mancini et al., 1998; Susin et al., 1999a) have been reported to be coreleased with cytochrome c in other systems, often in the absence of a permeability transition.

Whereas proteins such as Bid, Bax, and certain endogenous X enonpus egg cytosolic factors produced only a limited outer membrane permeability, we detected another cytosolic activity that caused a much stronger permeabilization effect. We termed this novel cytosolic activity PEF, as it enhanced, rather than initiated, outer membrane permeabilization (Figs. 5-10). The initial permeability caused by cytochrome c releasing factors is limited as it allows a relatively small cytochrome c flux across the outer membrane. This flux is sufficient to allow the loss of endogenous intermembrane space proteins, but not the rapid exchange of exogenous cytochrome c measured as accessibility of complexes III and IV (Fig. 5). However, if fresh cytosol (PEF) is present when cytochrome c-releasing factors act on mitochondria, an additional mitochondrial change occurs which strikingly enhances the permeability of the outer membrane to exogenous cytochrome c. From measurements of complex IV accessibility such as those presented in Fig. 4, we calculate that 1 μl of apoptotic mitochondrial can oxidize ~60 nmol of cytochrome c per minute. A similar volume of X enonpus egg mitochondria contains ~12 pmol of cytochrome c, we conclude that the rate of cytochrome c exchange across the outer membrane produced by PEF is at least 5,000-fold greater than that needed for complete efflux of endogenous cytochrome c per minute.

A potential explanation for the PEF activity we observed is that PEF could be an analogue of Bax or Bid that is present in cytosol at higher concentrations than those we used with recombinant Bid and Bax. However, this notion can be ruled out for the following reasons: (a) when cytosol activated by pretreatment with cytochrome c is subsequently mixed with mitochondria, rapid release of cytochrome c is observed, likely due to the cleavage and activation of endogenous X enonpus Bid. This activated cytosol, however, has lost PEF activity rather than gained it; (b) recombinant tBid was added at five-fold higher concentrations (170 ng/ml) than those required for cytochrome c release (not shown); and (c) most compelling PEF-containing cytosol does not possess the ability to cause rapid release of cytochrome c, and therefore cannot contain Bid or Bax activity greater than the amounts of recombinant protein we added. Further dilution of PEF-con-
The high permeability caused by PEF is consistent with a major rearrangement of the outer mitochondrial membrane, as a similar permeability (complex III or IV accessibility) was obtained with Triton X-100 (Figs. 7-9), osmotic breakage of the outer membrane (Fig. 6), and with digitonin (Fig. 7) in the absence of cytosol. The permeability increase by PEF was not, however, accompanied by observable changes in mitochondrial ultrastructure (Fig. 2, B and E). Furthermore, evidence suggests that the inner mitochondrial membrane remains intact even after PEF has acted to permeabilize the outer membrane. We observed that the mtHsp70 protein was released from the matrix of Xenopus mitochondria treated with certain concentrations of the detergent digitonin but was not released with apoptosis (Fig. 7), in agreement with another report that the inner membrane remains impermeable to proteins (Kohler et al., 1999). In addition, our earlier data (Kluck et al., 1997a) show that inner membrane potential (as measured by mitochondrial retention of the dye, DiOC6) remains high for at least 3 h after cytochrome c release, a finding inconsistent with inner membrane permeabilization. Thus, the effects of tBid, Bax, and PEF are confined to the outer membrane, rather than reflecting a global alteration in mitochondrial integrity.

The outer membrane permeability changes we observed were not due to a feedback effect of cytochrome c-activated caspases. Caspase inhibitors failed to block cytochrome c and AK efflux induced by Bid, Bax and the pro-apoptotic proteins in Xenopus cytosol. Moreover, Bid and Bax induced cytochrome c release from mitochondria incubated in buffer alone (Fig. 5). Caspase inhibitors also did not block the permeabilizing activity of PEF, but rather enhanced it (Fig. 9).

Preliminary characterization showed that PEF is proteinaceous and apparently large (>300 kD). While the slow kinetics of PEF action may suggest enzymatic behavior, PEF is not a caspase, as it was not inhibited by caspase inhibitors. Indeed, PEF is inactivated by caspases, because cytosol in which caspasess had been activated by the addition of caspase inhibitors contained no PEF activity, and caspase inhibitors prolonged the effects of PEF in fresh cytosol. A PEF-like activity was also detected in human Jurkat cell extracts (not shown). It is possible that PEF is active in whole cells, as a similar assay monitoring oxygen consumption (complex IV activity) in permeabilized cells found that apoptosis induced high oxygen consumption in the presence of exogenous reduced cytochrome c (Vander Heiden et al., 1997).

There are several mechanisms by which pro-apoptotic Bcl-2 family members might induce outer membrane permeability without affecting the inner membrane. These membrane-associated proteins may form channels large enough for the passage of soluble proteins (reviewed by Reed, 1997) or cooperate with channels already present (Mannella, 1992) such as VDAC (Shimizu et al., 1999; Vander Heiden et al., 1997). A lternate mechanisms might be similar to the manner in which 15-lipoxygenase can interact specifically with organellar membranes to cause visible changes (3–10 nm) in the lipid structure and permeability to high molecular mass proteins (van Leyen et al., 1998), or to that in which Bax causes instabilities in artificial membranes (Basanez et al., 1999). It is unclear is the mechanism of PEF action, in particular how PEF is permitted to function only after a prior permeabilization of the outer membrane by factors such as Bid and Bax. One possibility is that PEF could interact with a target located on the luminal side of the outer membrane. In this case, PEF would only gain access to its sites of action when the outer membrane becomes permeable to some degree. Alternatively, PEF could require the cooperation of a non-soluble cofactor that is located within the intermembrane space (the experiment in Fig. 5 D rules out the involvement of a soluble intermembrane space protein). Yet another possibility is that PEF interacts directly with channels produced by tBid or Bax.

It is thought that eukaryotic mitochondria originate from a symbiotic relationship between primitive host cells and primitive gram-negative bacteria, which allowed the host cells to use oxygen to produce energy (Brown and Doolittle, 1997; Martin and Muller, 1998). It can be argued that pressures to maintain this symbiotic relationship have led to the utilization of death pathways involving mitochondria, i.e., apoptosis via cytochrome c or necrosis via reactive oxygen species (Kobayashi, 1998; Blackstone and Green, 1999). From this perspective, it is interesting to consider whether the Bcl-2 family might have evolved from primitive antibiotics produced by the host cell. All known effects of the Bcl-2 family members, with the exception of Bcl-2 cell cycle effects (Huang et al., 1997), can be linked to their regulation of cell death, often via effects on mitochondria. Indeed, the structures of Bcl-x (Muchmore et al., 1996; Sattler et al., 1997) and BID (Chou et al., 1999; MCDonnell et al., 1999) reveal similarities to those of the pore-forming domains of bacterial toxins. Moreover, like bacterial toxins, Bcl-2 proteins form channels in synthetic lipid membranes.

Other factors, derived from the host cell, might also have evolved to destroy mitochondrial integrity. Such products include defensins (White et al., 1995), granulysin (Stenger et al., 1998), bacterialicidal permeability-increasing proteins (Beamer et al., 1998), and channel-forming peptides (Nicolaity et al., 1994; B. Eechinger, 1997), such as mastoparan and alamethicin, both of which we have shown can release mitochondrial cytochrome c. PEF can now be added to this list of molecules affecting mitochondrial permeability.

What are the implications of PEF activity for a cell? If PEF is present, attempts to rescue doomed cells by using caspase inhibitors would likely be futile, and may even enhance mitochondrial damage by leaving PEF fully active. Some cells given an apoptotic stimulus undergo necrotic death instead of apoptosis when caspase inhibitors are present (Green and Reed, 1998), and in such cases PEF might be responsible for ensuring that mitochondria are irreversibly damaged. On the other hand, in a para-1 and caspase-9 knockout mice, where the cytochrome c-initiated caspase activation pathway is missing, many neurons survive despite likely mitochondrial cytochrome c release (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998). Moreover, in the presence of caspase inhibitors, trophic factor-deprived primary sympa-
thletic neurons survive for many days despite mitochondrial cytochrome c translocation (Deshmukh and Johnson, 1998; M. Artinou et al., 1999). In such saved cells, neuronal mitochondria can survive the loss of cytochrome c and, upon restoration of trophic support, actually recover their content of cytochrome c after 24 h (M. Artinou et al., 1999). It has not yet been determined whether the recovery of mitochondrial cytochrome c in these neurons is due to multiplication of a few intact mitochondria, or to the reimport of (apo)-cytochrome c into mitochondria that recover from their permeabilization. We hypothesize that in most nonneural tissues, PEF provides a backup mechanism for killing the cell in the event of a disabled caspase pathway. This kind of redundancy in death pathways might be desirable in lymphocytes and Xenopus eggs, but not in irreplacable cells such as neurons in the adult organism.

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