Preservation of Mitochondrial Structure and Function after Bid- or Bax-mediated Cytochrome c Release

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Abstract. Proapoptotic members of the Bcl-2 protein family, including Bid and Bax, can activate apoptosis by directly interacting with mitochondria to cause cytochrome c translocation from the intermembrane space into the cytoplasm, thereby triggering A paf-1-mediated caspase activation. Under some circumstances, when caspase activation is blocked, cells can recover from cytochrome c translocation; this suggests that apoptotic mitochondria may not always suffer catastrophic damage arising from the process of cytochrome c release. We now show that recombinant Bid and Bax cause complete cytochrome c loss from isolated mitochondria in vitro, but preserve the ultrastructure and protein import function of mitochondria, which depend on inner membrane polarization. We also demonstrate that, if caspases are inhibited, mitochondrial protein import function is retained in UV-irradiated or staurosporine-treated cells, despite the complete translocation of cytochrome c. Thus, Bid and Bax act only on the outer membrane, and lesions in the inner membrane occurring during apoptosis are shown to be secondary caspase-dependent events.

Key words: apoptosis • mitochondria • membrane potential • protein import • electron microscopy

Introduction

The release of cytochrome c from mitochondria is central to many forms of apoptosis and promotes the A paf-1-mediated activation of effector caspases (Liu et al., 1996; Kluck et al., 1997a,b; Li et al., 1997; Y ang et al., 1997; Bossy-Wetzel et al., 1998). Cytochrome c release, whose mechanism is not yet understood, is known to be regulated by Bcl-2 family proteins. A ntiapoptotic members of this family, including Bcl-2 and Bcl-xL, bind to the mitochondrial outer membrane and block cytochrome c efflux (Kluck et al., 1997a; Y ang et al., 1997). In contrast, proapoptotic members of the B cl-2 family proteins, such as Bax, Bid, and Bak, promote the release of cytochrome c and other proteins of the mitochondrial intermembrane space (E skes et al., 1998; J urgensmeier et al., 1998; L ou et al., 1998; D esagher et al., 1999; Finucane et al., 1999; Kluck et al., 1999). To explain this protein release, various mechanisms have been suggested, some of which involve swelling of the mitochondrial matrix and subsequent mechanical rupture of the outer mitochondrial membrane. Matrix swelling is an osmotic effect proposed to be caused by the opening of a permeability transition (PT) pore in the inner membrane (M arzo et al., 1998; N arita et al., 1998; P astorino et al., 1998), or by hyperpolarization of the inner membrane (V ander Heiden et al., 1997). However, in many instances of apoptosis, mitochondrial swelling is not observed (e.g., Searle et al., 1975; M ancini et al., 1997; Z huang et al., 1998; M artinou et al., 1999) and several studies have reported that PT-related changes in the mitochondrial membrane potential (ΔΨm), as measured by the retention of potential-sensitive fluorescent dyes, either fail to occur in apoptosis or occur only downstream of the activation of effector caspases (Kluck et al., 1997a; Y ang et al., 1997; Bossy-Wetzel et al., 1998; E skes et al., 1998; Finucane et al., 1999; G oldstein et al., 2000).

These observations raise important questions: how much of mitochondrial function is disrupted during the process leading to cytochrome c release? Is a cell committed to die from mitochondrial dysfunction after cytochrome c re-
lease? One of the most critical mitochondrial activities is the import of proteins from the cytoplasm. Because most mitochondrial proteins are encoded by nuclear genes, protein import is essential, both for energy metabolism and for other essential functions such as amino acid degradation, steroid biosynthesis, and heme biosynthesis. For a cell to survive for extended periods after cytochrome c release, as do NGF-deprived primary sympathetic neurons rescued by caspase inhibitors (Deshmukh and Johnson, 1998; Neame et al., 1998; M artinou et al., 1999), these import-dependent functions are likely to be required.

Mitochondrial protein import involves an elaborate machinery (Pfanner and Meijer, 1997). Precursor proteins first bind to receptors at the outer membrane, and are then translocated through the TOM (translocase of the outer mitochondrial membrane) pore complex. Next, this outer membrane complex must join with the TIM (translocase of the inner mitochondrial membrane) pore complex on the inner membrane, allowing the precursor to cross into the mitochondrial matrix. The transfer across the inner membrane, which is accompanied by the removal of the presequence by matrix processing proteases, is strictly dependent on the inner membrane potential, $\Delta \Psi_{m}$, and the ATP-dependent action of matrix Hsp70.

Because this process is well understood, we used import competence as a measure of the intactness and energetic state of the mitochondria. Our results show that the proapoptotic proteins, Bid and Bax, can induce the translocation of cytochrome c through the outer mitochondrial membrane without affecting mitochondrial protein import. Thus, Bid and Bax produce only subtle changes in mitochondria, preserving $\Delta \Psi_{m}$ despite allowing complete cytochrome c efflux through the outer membrane. In addition, electron microscopy revealed that the structure of mitochondria was not detectably changed, even when the mitochondria had lost all of their cytochrome c. These results are clearly incompatible with mechanisms for cytochrome c release based on permeability transition and subsequent mitochondrial swelling. Similarly, studies with intact cells exposed to apoptotic stimuli also revealed that under conditions where caspases are inhibited, mitochondrial protein import is preserved. Our findings that the direct effects of Bid and Bax on mitochondria are relatively benign, and that mitochondrial import can be maintained even in apoptotic cells when caspases are blocked, suggest that the mitochondrial process leading to cytochrome c release may not always be an irreversible commitment point for cell death.

**Materials and Methods**

**Buffers**

Mitochondrial isolation buffer (M1B) was made from the following reagents: 210 mM mannitol, 60 mM sucrose, 10 mM KCl, 10 mM sodium succinate, 5 mM EGTA, 1 mM ADP, 0.5 mM DTT, and 20 mM Hepes-KOH, pH 7.5. PT buffer (Halestrap et al., 1986) was comprised of the following: 125 mM KCl, 2.5 mM potassium phosphate, 2.5 mM sodium succinate, 2 mM NaADH, 20 mM Hepes-KOH, pH 7.4. Import buffer was made from the following reagents: 250 mM sucrose, 80 mM KCl, 5 mM MglCl, 2.5 mM sodium succinate, 2 mM NaADH, and 20 mM Hepes-KOH, pH 7.4.

**Mitochondrial Preparations**

Mitochondria were isolated from Xenopus eggs as previously described (Newmeyer et al., 1994; Newmeyer, 1998; von Aksen and Newmeyer, 2000).

**Mitochondrial Isolation from Human Myeloid HL-60 Cells.** Cells were grown to log phase in RPMI 1640 with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. 10$^6$ cells were washed in PBS twice, resuspended in 2 ml M1B including a protease inhibitor cocktail (“Complete”; Boehringer Mannheim) and lysed with 100 strokes of a Teflon homogenizer. A $\alpha$-tuberlin, unbroken cells and nuclei were pelleted at 200 g for 5 min, and the supernatant was centrifuged for 10 min at 5,000 g to pellet the mitochondria. A $\beta$-tuberlin resuspension in M1B, the mitochondrial protein content was estimated by absorbance at 280 nm in the presence of 0.5% SDS.

**Recombinant Bid and Bax**

Bax was prepared as previously described (Finucane et al., 1999). The cDNA coding for full-length human Bid was cloned into pGEX-4T1 (Amersham Pharmacia Biotech). Amino acids 57-62 were replaced by the thrombin cleavage sequence LVPRGS using site-directed mutagenesis (overlap extension method). The resulting fusion protein was activated by thrombin cleavage, producing the same COOH-terminal fragment of Bid that results from caspase-8 cleavage of wild-type full-length Bid. In addition, a 6-histidine tag was attached to the COOH terminus to facilitate purification of the active fragment.

The plasmid was transformed into Escherichia coli BL21 (DE3) (Invitrogen). A 1-liter culture was grown to an OD of 1, expression was induced by the addition of 0.5 mM IPTG, and the cells were harvested after two more hours of growth. The bacterial pellet was lysed by sonication in PBS containing 0.5% Triton X-100, 1 mM EDTA, 0.5 mM PMSF, and 10 $\mu$g/ml each of aprotonin and leupeptin. The lysate was spun for 30 min at 15,000 g, and the supernatant was filtered through a 0.22-$\mu$m filter and incubated for 2 h at 4°C with glutathione-Sepharose-4B (Amersham Pharmacia Biotech). A $\beta$-tuberlin wash each with lysis buffer containing 0.1% Triton X-100 and PBS, the beads were incubated with 100 U of thrombin in 4 ml PBS for 2 h at 22°C to cleave off the COOH-terminal portion corresponding to $\beta$-tuberlin (amino acids 61-195) with a 6H tag. The supernatant of the cleavage reaction, containing $\beta$-tuberlin, was bound to 4 ml Ni-NTA resin. This resin was loaded into a column and washed sequentially with PBS, PBS containing 300 mM additional NaCl, and finally PBS, pH 6.0, containing 300 mM NaCl. The $\beta$-tuberlin was eluted with 100 mM imidazole in PBS, pH 6.0, containing 300 mM NaCl and dialyzed against PBS containing 10% glycerol for 6 h before storage at −80°C.

**Protein Import Assay**

A fusion protein (Su9-DHFR) containing the mitochondrial targeting sequence of $\beta$-tuberlin was fused to dihydrofolate reductase (Pfanner et al., 1987) was used as a model substrate for protein import. The protein was synthesized and labeled by in vitro translation in reticulocyte lysate (Promega) in the presence of [$^{35}$S]methionine. For import, the precursor protein was added to isolated mitochondria or to permeabilized HeLa cells in buffer as indicated in the figure legends and the mixture incubated for 30 min at 22°C. Import reactions were stopped by 1 $\mu$M valinomycin and chilled on ice. A $\alpha$-tuberlin, mitochondria were preincubated for 5 min with 1 $\mu$M valinomycin to dissipate the membrane potential. Where indicated, protease K (200 $\mu$g/ml) was added for 10 min on ice to digest nonimported protein, and then inactivated by PM SF (1 $\mu$M) in a further 5-min incubation. Mitochondria were reisolated, and the precursor, intermediate, and mature forms of the labeled protein were resolved by SDS-PAGE and autoradiography.

**Mitochondrial Cytochrome c Release Assay**

Mitochondria were incubated as described in the figure legends and resolated by centrifugation for 10 min at 13,000 g. Supernatants were carefully removed, and the mitochondrial pellet was analyzed for cytochrome c content by SDS-PAGE and Western blotting, using a monoclonal anticytochrome c antibody (clone 7H 8.2C12, Pharmingen).

**Mitochondrial Inner Membrane Potential**

Mitochondria (0.3 mg/ml protein) or HeLa cells (10$^6$ cells/ml) were incubated in buffer containing 50 mM TMRE (tetramethylrhodamine ethyl-
ester) or 0.5 μM rhodamine-123 for 10 min at 22°C (mitochondria) or 37°C (cells), and the dye retention was analyzed by flow cytometry (FAC-Scan; Becton Dickinson) of the mitochondrial or cell suspensions. To measure background fluorescence, 1 μM valinomycin was added to the samples to dissipate ΔΨm. In Figs. 2 D and 5 D, this background was subtracted from all measurements when calculating the median fluorescence intensity. Qualitatively similar results were obtained with TMRE and rhodamine-123.

Mitochondrial Swelling

X enopus or HL60 mitochondria (0.3 mg/ml protein) were incubated in 1 ml of PT buffer for 30 min at 22°C after the addition of 10 μg/ml tBid, 50 μg/ml Bax, 1 mM CaCl2, and 20 μg/ml wash in KClosm, washed and postfixed in 1% osmium tetroxide, 3% potassium ferricyanide in 0.1 M sodium cacodylate, pH 7.4 for 1 h on ice. Samples were washed in ice-cold double-distilled H2O, en bloc-stained with ice-cold 1% aqueous uranyl acetate overnight. Subsequently, samples were washed in ice-cold double-distilled H2O, dehydrated in acetone, and embedded in Durcupan ACM resin. Electron microscopy and tomography were performed as detailed elsewhere (Perkins et al., 1997a, 1998). Volume segmentation techniques and methods for defining and dissecting components of the structure were used to facilitate interpretation and measurement (Perkins et al., 1997b). The volume was segmented into regions bounded by the outer, inner, and cristal membranes. Note that the inner boundary and cristal membranes are continuous surfaces but were segmented independently to examine cristal topography and connectivity.

Results

A n important question concerns whether apoptotic mitochondria become injured in a way that might be lethal for the cell, even if caspases are somehow inactivated. To begin to examine this issue, we used mitochondrial protein import as a measure of the functional integrity of these organelles. Intact, nonapoptotic, X enopus egg mitochondria imported a model precursor protein, Su9-DHFR, which was processed to the predicted sizes of intermediate and mature (imported) forms of the protein (Fig. 1 B, lane 6). Furthermore, the mature form was accumulated within mitochondria and, thus, protected against externally added protease (Fig. 1 B, lane 5). A s expected, transport was abrogated by treatment with the K+ ionophore valinomycin, which dissipates the inner membrane potential (Fig. 1 B, lanes 7 and 8). Thus, Su9-DHFR was imported into X enopus egg mitochondria in an authentic manner, as seen previously with Neurospora crassa mitochondria (Pflanner et al., 1987). Interestingly, incubation of X enopus egg mitochondria with a recombinant activated Bid protein (tBid), consisting of the COOH-terminal fragment of Bid corresponding to that produced by caspase-8 cleavage, caused complete release of mitochondrial cytochrome c within 30 min, but had no effect on the import of Su9-DHFR (Fig. 1 A and B). Because protein import is known to be dependent on mitochondrial membrane potential, this result implies that ΔΨm remained intact throughout the process leading to cytochrome c release. An important corollary of this result is that the event known as PT (for review see Zoratti and Szabo, 1995) is not required for tBid- or Bax-induced cytochrome c release (see Discussion). To determine whether this retention of protein import function was merely transient, we assessed import-competence in time course experiments. The results showed that mitochondria remained fully competent to import this precursor for at least 4 h after the loss of cytochrome c was complete (Fig. 1 C and D). A similar preservation of mitochondrial import was observed after cytochrome c release induced by treatment with recombinant Bax (Fig. 1 D). Thus, tBid and Bax did not affect mitochondrial protein import, despite the fact that these proteins permeabilized the outer mitochondrial membrane to an extent permitting cytochrome c (and other proteins; Kluck et al., 1999) to escape into the cytosol. The same results were obtained with both physiological buffers (e.g., import buffer; not shown) and with a special buffer designed to facilitate the permeability transition (PT buffer; Halestrap et al., 1986; Fig. 1 C).

Electron Microscopy and Tomography

Mitochondrial suspensions (1.5 mg/ml protein) were fixed by the addition of 1 vol of fixing buffer (4% glutaraldehyde, 2% low melting point agarose; Becton Dickinson) in 0.1 M sodium cacodylate buffer, pH 7.4. A titer fixation for 2 h on ice, samples were washed in cacodylate buffer and postfixed in 1% osmium tetroxide, 3% potassium ferricyanide in 0.1 M sodium cacodylate, pH 7.4 for 1 h on ice. Samples were washed in ice-cold double-distilled H2O and en bloc-stained with ice-cold 1% aqueous uranyl acetate overnight. Subsequently, samples were washed in ice-cold double-distilled H2O, dehydrated in acetone, and embedded in Durcopan ACM resin. Electron microscopy and tomography were performed as detailed elsewhere (Perkins et al., 1997a, 1998). Volume segmentation techniques and methods for defining and dissecting components of the structure were used to facilitate interpretation and measurement (Perkins et al., 1997b). The volume was segmented into regions bounded by the outer, inner, and cristal membranes. Note that the inner volume remained intact throughout the process leading to cytochrome c release. An important corollary of this result is that the event known as PT (for review see Zoratti and Szabo, 1995) is not required for tBid- or Bax-induced cytochrome c release (see Discussion). To determine whether this retention of protein import function was merely transient, we assessed import-competence in time course experiments. The results showed that mitochondria remained fully competent to import this precursor for at least 4 h after the loss of cytochrome c was complete (Fig. 1 C and D). A similar preservation of mitochondrial import was observed after cytochrome c release induced by treatment with recombinant Bax (Fig. 1 D). Thus, tBid and Bax did not affect mitochondrial protein import, despite the fact that these proteins permeabilized the outer mitochondrial membrane to an extent permitting cytochrome c (and other proteins; Kluck et al., 1999) to escape into the cytosol. The same results were obtained with both physiological buffers (e.g., import buffer; not shown) and with a special buffer designed to facilitate the permeability transition (PT buffer; Halestrap et al., 1986; Fig. 1 C).

It could have been argued that X enopus egg mitochondria are simply unable to undergo a PT and are, thus, atypical. However, as Fig. 2 shows, these mitochondria exhibited the classic signs of PT when treated with Ca2+ valinomycin, or mastoparan (a wasp venom peptide that has previously been employed as a PT-inducing agent; Pfeiffer et al., 1995). To measure PT, we examined several parameters. First, we assessed large amplitude mitochondrial swelling, which is reflected by a decrease in A 520 (Fig. 2 A). Second, we measured the loss of ΔΨm, using...
flow cytometry to assay the retention of the potential-sensitive dyes, TMRE (Fig. 2, D and E) and rhodamine-123 (not shown), in individual mitochondria. Finally, we assayed the import of Su9-DHFR (Fig. 2 C). Mitochondrial import, although interesting in its own right, also serves as an independent indicator of inner membrane polarization.

As shown by each of these measurements, PT occurred in response to treatment with Ca$^{2+}$, mastoparan, or valinomycin, but not tBid or Bax. The loss of cytochrome c or other intermembrane space proteins also has been reported in the context of PT (Narita et al., 1998; Pastorino et al., 1998; Susin et al., 1999), and we observed complete release of cytochrome c (Fig. 2 B) in response to mastoparan and valinomycin. However, with Ca$^{2+}$-induced PT, cytochrome c was almost entirely retained by mitochondria (Fig. 2 B; a small amount of cytochrome c was detected in the supernatants, not shown). Based on this result, and the A$_{520}$ measurements (Fig. 2 A), it seems that Ca$^{2+}$ swells mitochondria to a lesser extent than the other PT-inducers, leaving most outer membranes intact.

In contrast, tBid and Bax caused a complete loss of cytochrome c but none of the manifestations of PT. In particular, mitochondrial protein import, which depends on $\Delta \Psi_{mt}$, was maintained at high levels after the addition of tBid or Bax. Moreover, we found that cyclosporin A, the most widely used inhibitor of PT, inhibited the loss of membrane potential and swelling of Xenopus mitochondria produced by moderate Ca$^{2+}$ concentrations (inhibition was nearly complete at 50 $\mu$M Ca$^{2+}$ and partial at 150 $\mu$M; Fig. 2 A).
We conclude, first, that the efflux of cytochrome c induced by tBid or Bax is not dependent on PT or swelling mitochondria were preincubated in PT buffer with or without 10 μM cyclosporin A (A) for 15 min at 22°C, and CaCl₂ was added at the indicated concentrations. After 30 min at 22°C, the mitochondria were reisolated and their cytochrome c content was analyzed by Western blotting.

3 A), but had no effect on tBid-induced release of cytochrome c, regardless of the concentration of tBid (Fig. 3 B). We conclude, first, that the efflux of cytochrome c induced by tBid or Bax is not dependent on PT or swelling and, second, that mitochondrial loss of cytochrome c is not sufficient to induce PT.

To confirm directly that large amplitude swelling and subsequent physical disruption of the outer membrane do not accompany apoptotic cytochrome c release, we used two ultrastructural approaches. Isolated Xenopus egg mitochondria, after treatment with tBid or Ca²⁺, were analyzed by both standard transmission electron microscopy (Fig. 4, A–C) and a three-dimensional reconstruction technique, termed electron tomography, that allowed us to visualize the entire outer and inner membrane topologies at a relatively high resolution (Fig. 4, D–I). No significant changes in ultrastructure were apparent after tBid treatment. In particular, all of the mitochondria displayed dense matrices and apparently intact outer membranes (Fig. 4, A, D, and G). In contrast, after the addition of Ca²⁺, all mitochondria exhibited larger and less dense matrices, which is consistent with PT-dependent matrix swelling (Fig. 4, C, F, and I). Despite this swelling, outer membranes appeared to remain intact, which is consistent with the observed retention of cytochrome c under these conditions (Fig. 2 B).

It could have been argued that these results obtained with Xenopus mitochondria were irrelevant to published studies using mammalian cells and organelles. However, as shown in Fig. 5, mitochondria isolated from nonapoptotic human HL-60 promyelocytes behaved much like the Xenopus mitochondria. Treatment of HL-60 mitochondria with tBid induced a rapid release of cytochrome c without swelling of mitochondria (as determined by A₅₅₀ changes) or dissipation of ΔΨₘ (as determined by flow cytometric analysis of TMRE uptake and import competence). A gain, we found that calcium and mastoparan treatment induced PT, leading to the loss of the membrane potential and the swelling of mitochondria (Fig. 5, A–D). As seen with mitochondria from Xenopus (Fig. 2), calcium led to only moderate swelling and minimal release of cytochrome c (Fig. 5 A and B), which was detectable in the supernatant only upon overexposure of Western blots (not shown). Thus, the classical PT does not by itself always produce significant cytochrome c release. A time course experiment showed that HL-60 mitochondria remained import-competent for at least 3 h after tBid-mediated cytochrome c release was complete (Fig. 5 E).

Next, we wanted to determine whether mitochondria in cultured cells would behave in a similar manner. First, using digitonin, we permeabilized nonapoptotic HEK 293 cells and induced apoptotic changes by the addition of recombinant Bid to the surrounding buffer. Fig. 6 shows that recombinant tBid, when added above a certain threshold amount, causes complete mitochondrial cytochrome c release from the mitochondria in these permeabilized cells, while preserving the ability of the mitochondria to import the precursor protein, at least within the time period examined. Thus, the mitochondria in permeabilized cells are similar to isolated mitochondria in their response to recombinant tBid.

Next, we examined HEK 293 cells undergoing UV-induced apoptosis. At 8 h after UV treatment, mitochondrial depolarization, as determined by flow cytometric measurement of rhodamine-123 retention in individual cells, had occurred in 67% of the cells (Fig. 7 A). However, depolarization was almost completely blocked by treatment with the caspase inhibitor zVAD-fmk, in agreement with earlier results (Bossy-Wetzel et al., 1998); only 5% of the cells showed a loss of ΔΨₘ. After this flow cytometric analysis, we assayed cytochrome c release and import competence of mitochondria in these cells after selective permeabilization of the plasma membrane with digitonin (Fig. 7 B). In UV-treated cells, cytochrome c was completely released from the mitochondria, regardless of whether caspases were inhibited by the addition of zVAD-fmk. Mitochondrial import competence was strongly decreased in the apoptotic sample; however, in the presence of the caspase inhibitor, import competence was retained to a substantial degree. This not only confirms that the loss of the membrane potential is a caspase-dependent event, but also extends to intact cells our finding (obtained with isolated mitochondria [Figs. 1, 2, and 5] and permeabilized cells [Fig. 6]) that mitochondria can maintain protein import function despite the complete loss of cytochrome c from the intermembrane space.

A similar result was obtained when HEK 293 cells were induced to undergo apoptosis by incubation with staurospo-
rine. After 20 h in the presence of staurosorine, ~70% of the cells showed an apoptotic phenotype, and the entire population had a strongly decreased ΔΨm. However, in the presence of zVAD-fmk, most cells retained a high membrane potential (Fig. 8 A). The staurosporine-treated cells had lost virtually all of their mitochondrial cytochrome c by this time, but the mitochondria remained import-competent when caspase activation was blocked (Fig. 8 B). As Bax is reported to mediate staurosporine-induced apoptosis through translocation to mitochondria and induction of cytochrome c release (Wolter et al., 1997; Desagher et al., 1999; Murphy et al., 2000), this result confirms, under physiological conditions, our findings with recombinant Bax and isolated mitochondria.

Discussion

Previous studies in our laboratory showed that cytochrome c release in the Xenopus cell-free system was not accompanied by reduced mitochondrial retention of certain fluorescent dyes that are thought to mirror the inner membrane potential (Kluck et al., 1997a). Similar findings were reported from studies in which potential-sensitive dyes were incubated with cultured cells undergoing apoptosis (Vander Heiden et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998; Eskes et al., 1998). Because PT is invariably associated with a loss of ΔΨm, these data argued that cytochrome c release in the Xenopus system, and in some cultured cell models, was independent of PT.
to confirm that based on the import of a mitochondrial precursor protein, we used a completely independent technique, measured dye uptake in individual isolated mitochondria (5 mg/ml as protein) were incubated in PT buffer, with the addition (as indicated) of 10 μg/ml tBid, 1 mM CaCl\(_2\), or 20 μg/ml mastoparan for 30 min at 22°C. (A) Mitochondrial swelling was assessed by comparing absorbance at 520 nm before and 25 min after the indicated additions were made. The ΔA\(_{520}\) decrease produced by mastoparan was taken as 100%. 30 min after additions, aliquots were removed and analyzed for mitochondrial cytochrome c content (B) or import competence (C); 50 nM TMRE was added to the remaining samples, and (D) dye uptake was measured by flow cytometry after a further 10 min at 22°C. Finally, a second measurement of background fluorescence was made after valinomycin (1 μM) was added to all samples. B shows the median fluorescence with this background subtracted. (E) HL-60 mitochondria remain import-competent for at least 3 h after complete cytochrome c release. HL-60 cell mitochondria (5 mg/ml protein) were incubated in PT buffer in the presence or absence of tBid (10 μg/ml) as indicated. A aliquots were taken at the indicated times and analyzed for cytochrome c content and import competence. A a control showing import dependence on ΔΨ\(_{m}\), 1 μM valinomycin was added where indicated. Data are representative of three independent experiments.

However, other groups have disagreed with these conclusions and, in particular, have questioned the incautious use of potential-sensitive dyes (e.g., Metivier et al., 1998). For example, artifacts can arise because of the self-quenching behavior of some dyes. Also, with whole cells, the plasma membrane potential can influence the amount of dye taken up by the cell. To minimize such artifacts, we used TMRE, a dye reported to be free from self-quenching and measured dye uptake in individual isolated mitochondria or permeabilized cells by flow cytometry. Furthermore, we used a completely independent technique, based on the import of a mitochondrial precursor protein, to confirm that ΔΨ\(_{m}\) remains intact after cytochrome c release and, thus, that PT is not required for the release of proapoptotic proteins from the intermembrane space (Figs. 1, 2, and 5–8).

Our electron microscopic analysis failed to detect any significant structural changes in Bax- or tBid-treated mitochondria. This rules out a potential mechanism for cytochrome c release involving the induction of permeability transition and subsequent large amplitude swelling. In this context, it is notable that Ca\(^{2+}\) could induce significant mitochondrial matrix swelling even without significant loss of cytochrome c, suggesting that the outer mitochondrial membrane is fairly resistant to rupture. Our observation that tBid-induced cytochrome c release was unaffected by cyclosporin A under conditions in which this drug blocked Ca\(^{2+}\)-induced PT (Fig. 3) is also completely inconsistent with a PT-based mechanism of cytochrome c release. Finally, the finding that a loss of the mitochondrial membrane potential in apoptotic HeLa cells can be prevented by caspase inhibition (Figs. 7 and 8; Bossy-Wetzel et al., 1998) proves that, in these cells, depolarization is a secondary event.

At what point in the apoptotic pathway does a cell become irreversibly committed to die? The answer to this important question may depend on the fate of mitochondria. A t issue is whether the events leading to cytochrome c release cause irreversible damage to mitochondria. Even in the absence of active caspases, such mitochondrial damage could lead to cell death, although this death might take the form of necrosis rather than apoptosis. Indeed, a shift from apoptotic to necrotic death has been demonstrated for interdigital cells in A pafl–1-null mice (Chautan et al., 1999), which are defective in cytochrome c-mediated caspase activation. On the other hand, some cell types appear to require caspases for death. For example, studies of Bax-mediated apoptosis in primary neurons showed that these cells can recover from cytochrome c translocation, provided that caspase activation is blocked (Deshmukh and Johnson, 1998; Neame et al., 1998; Martinou et al., 1999). Similarly, some cell types in A pafl-1 or caspase-9 nullizygous mice survive abnormally, despite the presumed release of mitochondrial cytochrome c (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998). Because cell survival presumably requires functioning mitochondria, this suggests that mitochondria whose outer membranes have been permeabilized by Bax (or a related protein) can be restored to normal function.

To address these issues, it is important to determine the extent of injury inflicted on mitochondria by proapoptotic proteins such as Bax and Bid. In the present study, we examined the effects of Bax and Bid, using refined techniques that have not previously been applied to apoptotic mitochondria. First, we showed that Bax and Bid did not...
compromise mitochondrial protein import, a primary indicator of mitochondrial integrity and function. Second, we examined mitochondrial ultrastructure, using both transmission electron microscopy and a three-dimensional electron tomographic reconstruction technique that is capable of displaying the membrane contours of an entire mitochondrion. Such imaging revealed that Bax and BID failed to alter the mitochondrial membrane and matrix ultrastructure, whereas Ca^{2+}-induced substantial swelling of the matrix (although without causing substantial outer membrane breakage or release of cytochrome c). Finally, we showed that, in the presence of the caspase inhibitor Z-VAD-fmk, HeLa cells treated with lethal doses of UV or staurosporine underwent a complete loss of mitochondrial cytochrome c, but retained a significant amount of mitochondrial protein import function.

Exactly how proteins like tBid and Bax permeabilize the outer mitochondrial membrane is not yet clear. Potential escape routes of cytochrome c include the following: the
VDAC channel (Shimizu et al., 1999); TOM 40, which forms a channel for protein import (Hill et al., 1998); or a protein channel formed de novo by Bcl-2 family proteins (Schendel et al., 1998). In any case, our results suggest that the mechanism may be sufficiently delicate and reversible to allow mitochondria to be rescued later, provided that downstream apoptotic effectors in the cell, such as caspases, are inactivated.

Although caspases can be inhibited by gene knockout or pharmacological reagents, there are also potential physiological mechanisms through which caspases could be inhibited in vivo. For example, IAP proteins could be activated, or components of the apoptosome, like caspase-9 or A paf-1, could be inactivated by postsynthetic modification or transcriptional downregulation. The importance of these postmitochondrial regulatory mechanisms should, in principle, be dependent on whether mitochondrial injury by itself is lethal for the cell. Our results show that pro- apoptotic Bcl-2 family proteins produce only subtle mitochondrial permeability, which is limited to the outer membrane. Such mild permeabilization would create a situation in which cytochrome c and other intermembrane space proteins are able to equilibrate between the cytoplasm and the mitochondrial intermembrane space, but mitochondrial matrix and inner membrane topology and composition are largely preserved.

Even if the integrity of the inner membrane is disturbed, how could mitochondria maintain their membrane potential despite the loss of cytochrome c from the intermembrane space? In intact apoptotic cells, even if respiration stops because of limited cytochrome c concentration, cellular ATP levels could be maintained by glycolysis, and $\Delta \Psi_m$ could be maintained at some level by proton extrusion, catalyzed by ATP synthase running in reverse (this is presumably how respiration-deficient p0 cells maintain some membrane potential). In contrast, our experiments with isolated mitochondria provided no external source of ATP. However, there are two reasons why isolated mitochondria with permeable outer membranes could maintain a high membrane potential: (1) there are no ATP-consuming metabolic processes outside the mitochondria; and (2) cytochrome c equilibrates from the intermembrane space to the entire buffer volume, resulting in a strong but not infinite dilution of cytochrome c. The residual concentration may sustain enough respiration to maintain a high membrane potential.

A similar situation may occur in intact cells after cytochrome c release and, indeed, the final cytochrome c concentration might be even higher in cells than in our in vitro experiments, because cytochrome c becomes diluted only within the relatively small volume of the cell. In intact Xenopus egg mitochondria, cytochrome c is present in roughly a 1.5-fold stoichiometric excess over other constituents of the respiratory chain (Kluck, R.M., and D.D. Newmeyer, unpublished data). However, as cytochrome c acts catalytically rather than stoichiometrically, this may correspond to a large functional excess. In support of this idea, we found in our experiments with permeabilized cells (Fig. 6) that if the cells were diluted greatly or washed, $\Delta \Psi_m$ was lost quickly (von Ahsen, O., and Newmeyer, D.D., unpublished results).

Our previous studies identified a cytosolic activity, PEF, that greatly increases the permeability of the mitochondrial outer membrane (Kluck et al., 1999). PEF does not act on intact mitochondria, but requires the prior permeabilization of the outer membrane by proapoptotic proteins such as Bax and Bid. The experiments in Figs. 1–5 were performed in the absence of cytosol, and therefore, bear no relevance to PEF. The permeabilized cells used in Fig. 6 could perhaps contain PEF, depending on the degree of solubility of this factor. However, the experiments shown in Figs. 7 and 8 used intact cells, which are expected to contain PEF. If PEF is active in these cells, we can conclude that PEF also does not compromise the protein import function of apoptotic mitochondria. Moreover, PEF is apparently not responsible for the caspase-dependent loss of membrane potential and protein import observed in Figs. 7 and 8, because PEF does not require caspases for its function and, indeed, is inactivated by caspases. The functions of PEF could be, first, to insure that the permeabilization of the outer mitochondrial membrane is irreversible and, second, to increase the accessibility of the respiratory chain to cytochrome c molecules that reenter from the cytoplasm, thus, allowing a higher respiratory rate and membrane potential.

As our results now show, the maintenance of a membrane potential in apoptotic cells allows mitochondria to sustain protein import function, at least for some time. In principle, a mechanism could exist in certain cell types to restore outer membrane integrity to mitochondria that have been permeabilized by Bax-like proteins, especially if PEF were absent or inactivated. This would allow these rescued mitochondria to reaccumulate cytochrome c and other intermembrane space proteins (note that import of cytochrome c does not depend on $\Delta \Psi_m$, but import of another intermembrane space protein, like sulfite oxidase, does; Zimmermann et al., 1981; Ono and Ito, 1984). If cells can somehow restore mitochondrial outer membrane integrity after the release of cytochrome c, then the possibility is raised that mechanisms for modulating the function of downstream caspases (e.g., through regulating the A paf-1/caspase-9 apoptosome) could in certain cases help determine the survival of cells.

We thank Drs. N. Pfanner for Su9-DHFR DNA, Tomomi Kuwana for Bid DNA, Nigel Waterhouse for helpful discussions, and Sunny Han (all three from La Jolla Institute for Allergy and Immunology) for technical assistance. This work was supported by the National Institutes of Health (NIH) grant GM 50284 to Donald D. Newmeyer and fellowship A H 761-1 from the Deutsche Forschungsgemeinschaft to O. von Ahsen. Some of the work included here was conducted at the National Center for Microscopy and Imaging Research at San Diego, which is supported by NIH grant RR 04050 to Mark H. Ellisman. C. Renken acknowledges support from NIH/NIGMS M BRS Program Grant R25-GM 58906 and additional support from Grant-in-Aid 98-256A from the Western States Affiliate of the American Heart Association to T.G. Frey. E. Bossy-Wetzel was supported by NIH training grant AG 00252-02. This is publication No. 346 from the La Jolla Institute for Allergy and Immunology.

Submitted: 17 March 2000
Revised: 8 June 2000
Accepted: 13 July 2000

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Title:
Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release

Date:
2000-09-04

Citation:
von Ahsen, O., Renken, C., Perkins, G., Kluck, R. M., Bossy-Wetzel, E. & Newmeyer, D. D. (2000). Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. JOURNAL OF CELL BIOLOGY, 150 (5), pp.1027-1036. https://doi.org/10.1083/jcb.150.5.1027.

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