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 Apaf-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 Apaf-1–mediated activation of effector caspases (Liu et al., 1996; Kluck et al., 1997a,b; Li et al., 1997; Yang 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. Antiapoptotic members of this family, including Bcl-2 and Bcl-x_L, bind to the mitochondrial outer membrane and block cytochrome *c* efflux (Kluck et al., 1997a; Yang et al., 1997). In contrast, proapoptotic members of the Bcl-2 family, such as Bax, Bid, and Bak, promote the release of cytochrome *c* and other proteins of the mitochondrial intermembrane space (Eskes et al., 1998; Jurgensmeier et al., 1998; Luo et al., 1998; Desagher 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)[1] pore in the inner membrane (Marzo et al., 1998; Narita et al., 1998; Pastorino et al., 1998), or by hyperpolarization of the inner membrane (Vander Heiden et al., 1997). However, in many instances of apoptosis, mitochondrial swelling is not observed (e.g., Searle et al., 1975; Mancini et al., 1997; Zhuang et al., 1998; Martinou et al., 1999) and several studies have reported that PT-related changes in the mitochondrial membrane potential ($\Delta\Psi_m$), 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; Yang et al., 1997; Bossy-Wetzel et al., 1998; Eskes et al., 1998; Finucane et al., 1999; Goldstein 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-

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[1]*Abbreviations used in this paper:* $\Delta\Psi_m$, mitochondrial membrane potential; MIB, mitochondrial isolation buffer; PT, permeability transition.

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; Martinou 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 (MIB) 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 NADH, 20 mM Hepes-KOH, pH 7.4. Import buffer was made from the following reagents: 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2.5 mM sodium succinate, 2 mM NADH, 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 Ahsen 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 μg/ml streptomycin. 10^8 cells were washed in PBS twice, resuspended in 2 ml MIB including a protease inhibitor cocktail ("Complete"; Boehringer Mannheim) and lysed with 100 strokes of a Teflon homogenizer. After dilution, unbroken cells and nuclei were pelleted at 200 *g* for 5 min, and the supernatant was centrifuged for 10 min at 5,500 *g* to pellet the mitochondria. After resuspension in MIB, 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 pGEX4T1 (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, 1 mM DTT, 0.5 mM PMSF, and 10 μg/ml each of aprotinin and leupeptin. The lysate was spun for 30 min at 15,000 *g*, and the supernatant was filtered through a 0.22-μm filter and incubated for 2 h at 4°C with glutathione–Sepharose-4B (Amersham Pharmacia Biotech). After three washes 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 tBid (amino acids 61–195) with a 6xHis tail. The supernatant of the cleavage reaction, containing tBid-His_6, 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. tBid 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 F_0-F_1 ATPase subunit 9 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 μM valinomycin and chilling on ice. As controls, mitochondria were preincubated for 5 min with 1 μM valinomycin to dissipate the membrane potential. Where indicated, proteinase K (200 μg/ml) was added for 10 min on ice to digest nonimported protein, and then inactivated by PMSF (1 μM) 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 reisolated 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 anti–cytochrome *c* antibody (clone 7H8.2C12; PharMingen).

Mitochondrial Inner Membrane Potential

Mitochondria (0.3 mg/ml protein) or HeLa cells (~10^6 cells/ml) were incubated in buffer containing 50 nM 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 (FACScan; Becton Dickinson) of the mitochondrial or cell suspensions. To measure background fluorescence, 1 μM valinomycin was added to the samples to dissipate $\Delta\Psi_m$. 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

Xenopus 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 $CaCl_2$, and 20 μg/ml wasp venom mastoparan (Sigma Chemical Co.) or buffer. Mitochondrial swelling was assessed by a decrease in absorbance at 520 nm (Knight et al., 1981; Jurgensmeier et al., 1998) using a Hitachi 2000 spectrophotometer.

Permeabilization of HeLa Cells

HeLa cells were permeabilized essentially as previously described (Görlich et al., 1994; Heibein et al., 1999). In brief, the cells were harvested by trypsinization, washed once with medium and twice with ice-cold PBS, and then resuspended in import buffer (this matches reasonably well the conditions described by Görlich et al. [1997] and Heibein et al. [1999]) at 10^6 cells/ml. Finally, digitonin was added to 50 μg/ml and cells were incubated for 5–10 min on ice. In most experiments, 100% of the cells were permeabilized after 5–7 min, as indicated by trypan blue staining. To stop the action of digitonin, 1% BSA was added and cells were washed in import buffer. Control experiments showed that the plasma membrane was selectively permeabilized, as cytosolic proteins were lost from the cell pellets while cytochrome *c* was retained. For cytochrome *c* release and import assays, permeabilized cells were incubated in import buffer.

Cell Culture and Induction of Apoptosis

HeLa cells were grown in DME (GIBCO BRL) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To induce apoptosis, cells were washed and covered with PBS and irradiated with ultraviolet C at 180 mJ/cm^2. After irradiation, PBS was removed and replaced by medium for further incubation. Staurosporine was used at 1 μM in medium for 20 h. Caspase inhibition was achieved by including 100 μM zVAD-fmk in the medium where indicated.

The mechanisms governing protein stability may respond in unforeseen ways to an apoptotic stimulus. Thus, it is possible for any particular housekeeping protein to change in abundance during apoptosis. Therefore, in the experiments shown in Figs. 7 and 8, gel loading was normalized by total protein amounts, and immunoblotting with control sera against actin and COX IV provided secondary controls. In the UV-treated cells, the actin levels remained high and COX IV levels were only slightly reduced. This may suggest that the mitochondrial content of UV-treated cells was somewhat reduced, perhaps because of autophagy. At worst, this would result in a slightly lower ratio of mitochondria to import substrate and, therefore, a slight underestimation of mitochondrial protein import. In staurosporine-treated cells, COX IV levels remained high, but actin was lost or degraded significantly, even in the presence of zVAD-fmk (not shown).

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 [Boehringer Mannheim] in 0.1 M sodium cacodylate buffer, pH 7.4). After 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 H_2O and en bloc–stained with ice-cold 1% aqueous uranyl acetate overnight. Subsequently, samples were washed in ice-cold double-distilled H_2O, stepwise-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 boundary and cristal membranes are continuous surfaces but were segmented independently to examine cristal topography and connectivity.

Results

An 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, *Xenopus* 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). As 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 *Xenopus* egg mitochondria in an authentic manner, as seen previously with *Neurospora crassa* mitochondria (Pfanner et al., 1987). Interestingly, incubation of *Xenopus* 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 $\Delta\Psi_m$ 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 *Xenopus* 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 Ca^{2+}, 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 $\Delta\Psi_m$, using

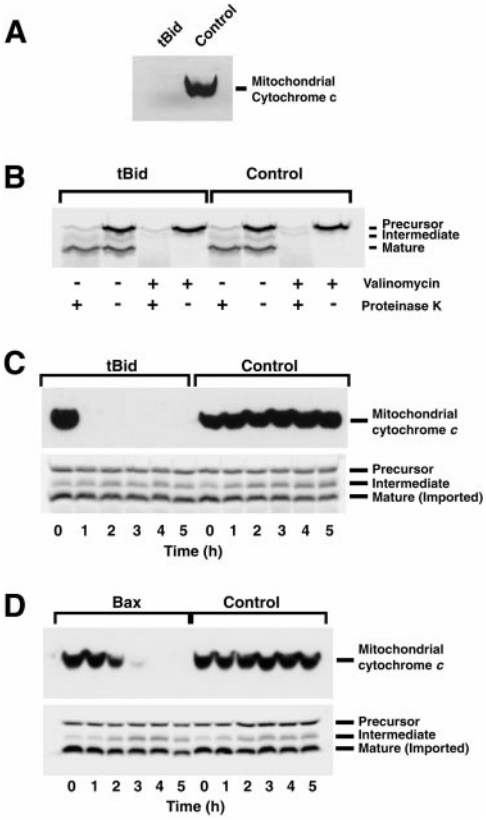

Figure 1. Protein import function is maintained by isolated mitochondria even after complete cytochrome *c* release. Mitochondria (1.5 mg/ml protein) were incubated in MIB containing 80 mM KCl, with or without 10 μg/ml tBid for 30 min at 22°C. (A) An aliquot was analyzed for mitochondrial cytochrome *c* content, showing a complete loss of cytochrome *c* from mitochondria incubated with tBid. (B) Mitochondria were reisolated and resuspended in import buffer containing 2 mM ATP, and the sample was divided into four aliquots. After a 5-min incubation in the presence or absence of valinomycin (a K^+ ionophore) to dissipate membrane potential, the precursor protein was added. After 30 min, the import reaction was stopped by the addition of valinomycin and cooling the samples on ice. Where indicated, proteinase K was added for 10 min to digest nonimported precursor. After addition of PMSF and a 5-min incubation on ice, mitochondria were reisolated and analyzed by SDS-PAGE and autoradiography. Inhibition of processing by valinomycin proves that protein import was dependent on membrane potential. Imported protein was processed to a mature size and protected against externally added protease. (C and D) Mitochondria remain import-competent for several hours after the complete loss of cytochrome *c* induced by tBid or Bax. Mitochondria (1.5 mg/ml) were incubated in PT buffer with or without the addition of 10 μg/ml tBid (C) or 50 μg/ml Bax (D). At the times indicated, aliquots were removed and analyzed for mitochondrial cytochrome *c* content (top) or import competence (bottom), as indicated by the processing of the precursor to a mature size by the mitochondrial matrix processing peptidase.

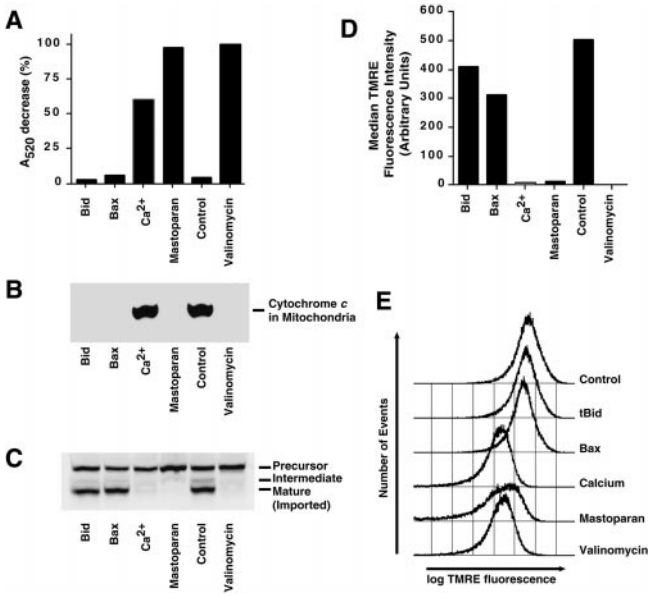

Figure 2. Bid-induced cytochrome *c* release does not depend on mitochondrial PT. Mitochondria (0.3 mg/ml as protein) were incubated in PT buffer, with the addition (as indicated) of 10 μg/ml tBid, 50 μg/ml Bax, 1 mM $CaCl_2$, 20 μg/ml mastoparan, or 1 μM valinomycin for 30 min at 22°C. (A) Mitochondrial swelling was assessed by absorbance at 520 nm measured before and 25 min after the indicated additions were made. The A_{520} decrease produced by valinomycin 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 and E) dye uptake was measured by flow cytometry after a further 10 min at 22°C. After this, valinomycin (1 μM) was added to all samples and a second measurement of background fluorescence was made. D shows the median fluorescence with this background subtracted (linear scale), while E shows histograms (log scale). Data are representative of five independent experiments.

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 $\Delta\Psi_m$, 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 μM Ca^{2+} and partial at 150 μM; Fig.

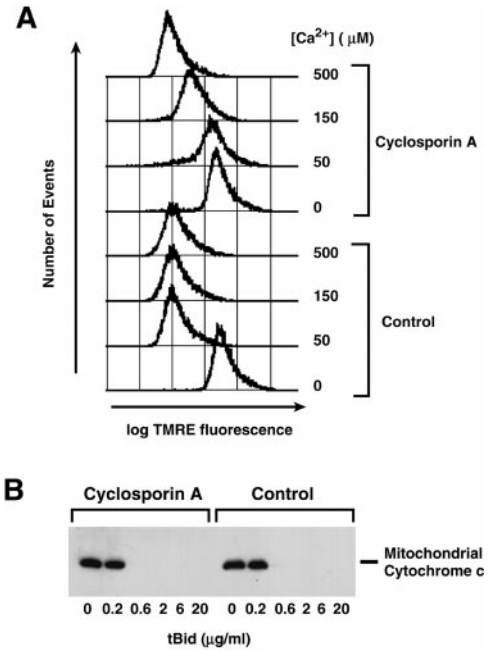

Figure 3. Cyclosporin A blocks PT in *Xenopus* mitochondria, but has no effect on tBid-induced cytochrome *c* release. (A) *Xenopus* mitochondria were preincubated in PT buffer with or without 10 μM cyclosporin A (A) for 15 min at 22°C, and $CaCl_2$ was added at the indicated concentrations. After 30 min, TMRE retention was measured by flow cytometry. (B) The lack of effect of cyclosporin A on tBid-induced cytochrome *c* release. *Xenopus* mitochondria were preincubated in PT buffer with or without 10 μM cyclosporin A for 15 min at 22°C, and tBid 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 Ca^{2+}, 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 Ca^{2+}, 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 A_{520} changes) or dissipation of $\Delta\Psi_m$ (as determined by flow cytometric analysis of TMRE uptake and import competence). Again, 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 HeLa 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 HeLa 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 $\Delta\Psi_m$. 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 HeLa cells were induced to undergo apoptosis by incubation with staurospo-

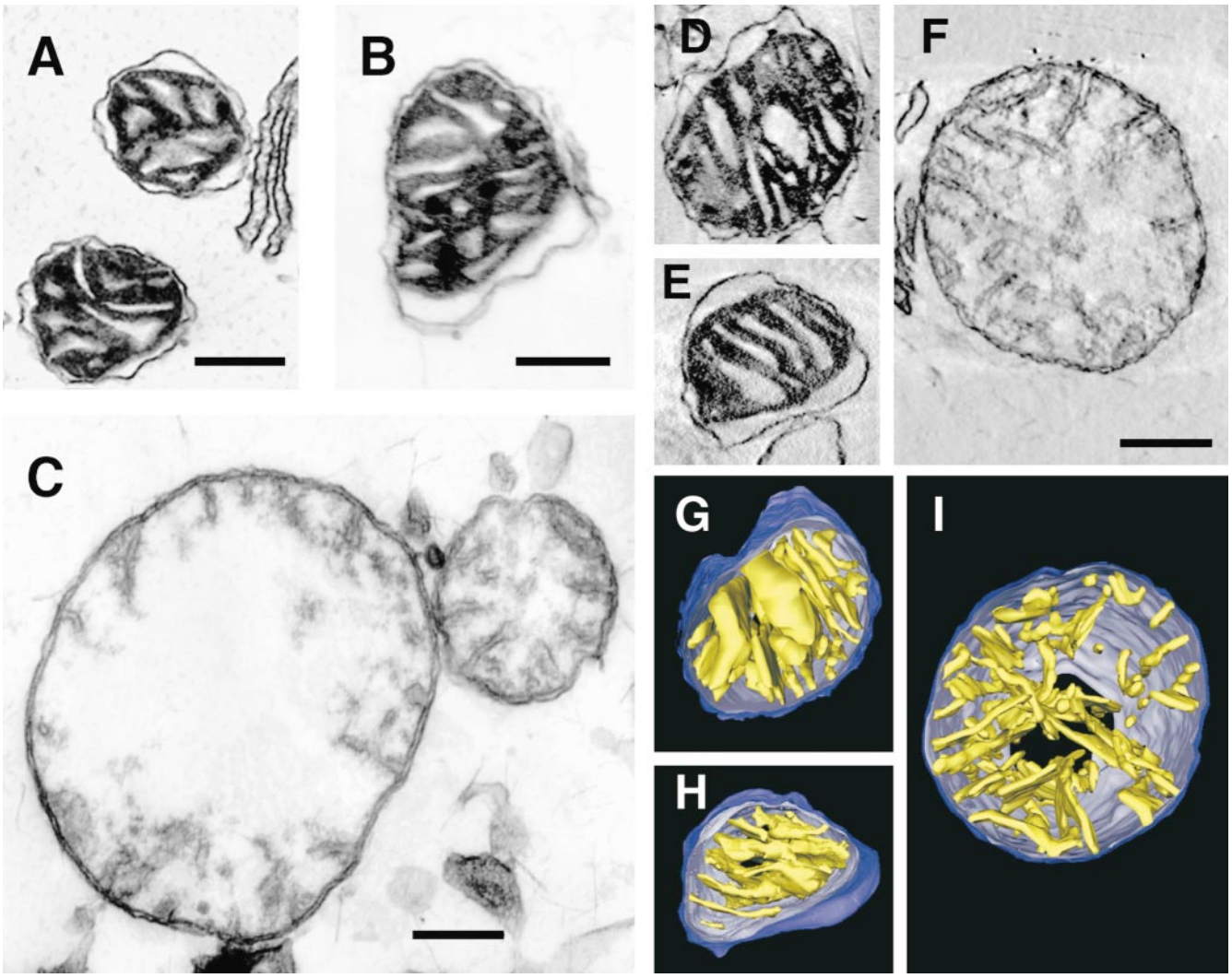

Figure 4. tBid produces no observable changes in mitochondrial ultrastructure, whereas Ca^{2+} causes swelling of the matrix. Shown are representative images from thin (~70 nm) sections using conventional electron microscopy. Before fixation, *Xenopus* mitochondria (0.3 mg/ml protein) were incubated for 30 min at 22°C in import buffer with (A) 10 μg/ml tBid, (B) buffer only, or (C) 1 mM $CaCl_2$. No significant difference between tBid-treated and control mitochondria could be observed (A and B). Only the calcium treatment led to matrix swelling (C). No breaks in outer membranes could be seen; even after calcium-induced swelling, the outer membranes apparently stay intact. Electron tomography also shows that tBid produces no significant structural alterations in mitochondria, whereas Ca^{2+} causes matrix swelling. D–F show cross-sections (2.3-nm thickness) through the middle of electron tomographic reconstructions of semi-thick (~500 nm) sections of mitochondria treated with 10 μg/ml tBid (D), buffer (E), or 1 mM $CaCl_2$ (F). Surface-rendered volumes of the same three mitochondria reconstructed for D–F are shown in G–I. Here, the outer membrane is shown in dark blue, the inner boundary membrane in light blue, and the cristal membranes in yellow. Bars, 250 nm.

rine. After 20 h in the presence of staurosporine, ~70% of the cells showed an apoptotic phenotype, and the entire population had a strongly decreased $\Delta\Psi_m$. 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 $\Delta\Psi_m$, these data argued that cytochrome *c* release in the *Xenopus* system, and in some cultured cell models, was independent of PT.

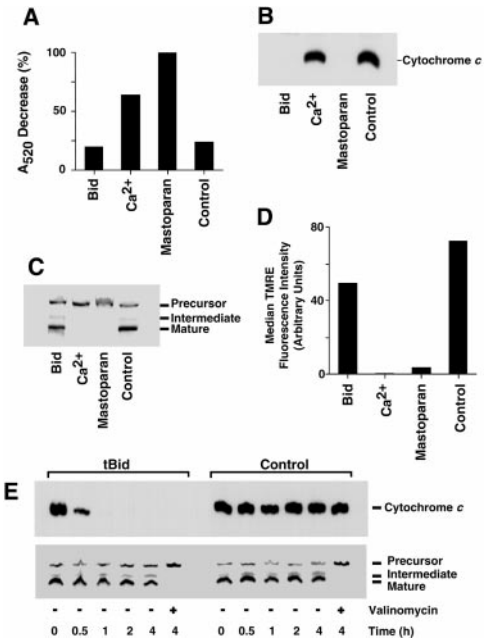

Figure 5. Mitochondria isolated from HL-60 cells retain protein import function and intact $\Delta\Psi_m$ after cytochrome *c* release. Mitochondria (5 mg/ml as protein) were incubated in PT buffer, with the addition (as indicated) of 10 μg/ml tBid, 1 mM $CaCl_2$, or 20 μg/ml mastoparan for 30 min at 22°C. (A) Mitochondrial swelling was assessed by comparing absorbance at 520 nm before and 25 min after the indicated additions were made. The A_{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. Aliquots of the reaction were taken at the indicated times and analyzed for cytochrome *c* content and import competence. As a control showing import dependence on $\Delta\Psi_m$, 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 $\Delta\Psi_m$ 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).

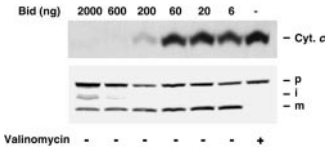

Figure 6. Bid releases cytochrome *c* from mitochondria in permeabilized HeLa cells without disruption of membrane potential or protein import. Permeabilized HeLa cells (2×10^5) were incubated in 100 μl of import buffer with the indicated amounts of Bid for 30 min at 22°C. An aliquot was spun down to analyze cytochrome *c* content, and the remaining sample was used to assess import competence. Cell pellets were extracted with NP-40 lysis buffer and analyzed as described in Fig. 7.

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. At 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 Apaf-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 Apaf-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

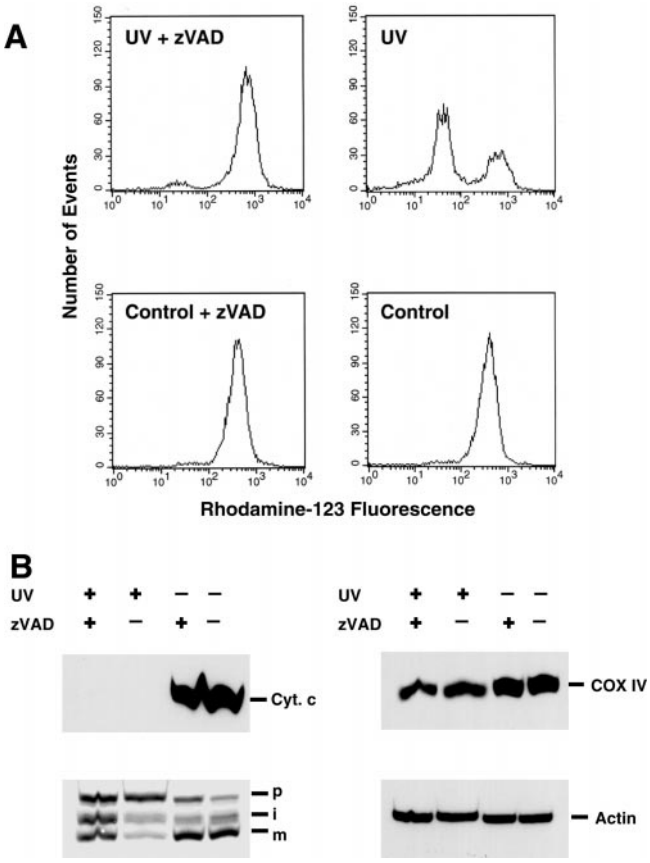

Figure 7. When caspases are inhibited, UV-irradiated HeLa cells display a complete loss of mitochondrial cytochrome *c*, but retain mitochondrial import function. (A) Loss of $\Delta\Psi_m$ in apoptotic cells is blocked by caspase inhibition. HeLa cells were exposed to 180 mJ/cm^2 UVC light and further cultured for 8 h in medium with or without 100 μM zVAD-fmk as indicated. After this time, >50% of UV-treated cells showed an apoptotic phenotype in the absence of zVAD-fmk. Cells were harvested, and rhodamine-123 retention was measured by flow cytometry (10,000 events shown without gating). In the presence of zVAD-fmk, apoptotic cells retain $\Delta\Psi_m$. (B) Mitochondrial import competence is retained in the presence of zVAD-fmk. After flow cytometric analysis (A), the cells were permeabilized with digitonin and analyzed for cytochrome *c* content and import-competence. Cell pellets were extracted with NP-40 lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM Tris-Cl, pH 8.0), and the protein content of the extract was determined by the Bradford assay. Finally, 50 μg of protein from each sample was subjected to SDS-PAGE and Western blotting. Quantitative analysis by phosphorimaging revealed 82 and 17% imported protein in the UV-treated samples with or without zVAD, respectively. Control samples gave 100 and 89% import, respectively (the control sample with zVAD was taken as 100%). Blots were stripped and reprobed with antibodies to COX-IV and actin to demonstrate equal gel loading.

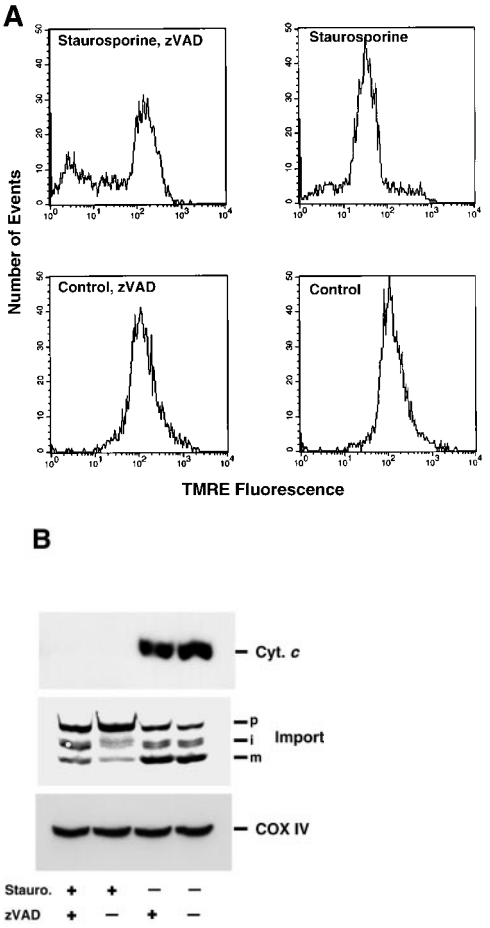

Figure 8. Mitochondrial import function remains partially intact in cells undergoing staurosporine-induced apoptosis, provided that caspases are inhibited. HeLa cells were cultured for 20 h in medium containing 1 μM staurosporine and/or 100 μM zVAD-fmk as indicated. Approximately 70% of staurosporine-treated cells were apoptotic at this time, whereas, in the presence of zVAD-fmk, 60% were intact and 40% necrotic. (A) Cells were harvested and TMRE uptake was assessed by flow cytometry (5,000 events shown without gating). Staurosporine-treated cells show a loss of $\Delta\Psi_m$, which is largely prevented by caspase inhibition. (B) Cytochrome *c* release and import competence were assessed after digitonin permeabilization as in Fig. 7. Quantitation by phosphorimaging revealed 38 and 13% imported protein in staurosporine-treated samples with or without zVAD-fmk. (Control samples gave 100% and 84%; import in the control sample with zVAD was defined as 100%.) The cytochrome *c* blot was stripped and reprobed with anti–COX-IV to demonstrate equal gel loading.

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 zVAD-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); TOM40, 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 Apaf-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 proapoptotic 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 undisturbed, 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 ρ_0 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 Apaf-1/caspase-9 apoptosome) could in certain cases help determine the survival of cells.

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