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Bid, Bax, and Lipids Cooperate to Form Supramolecular Openings in the Outer Mitochondrial Membrane

Tomomi Kuwana,[1] Mason R. Mackey,[2] Guy Perkins,[2] Mark H. Ellisman,[2] Martin Latterich,[3] Roger Schneiter,[4] Douglas R. Green,[1] and Donald D. Newmeyer[1,5]

[1]La Jolla Institute for Allergy and Immunology
10355 Science Center Drive
San Diego, California 92121

[2]National Center for Microscopy and Imaging Research
University of California, San Diego
La Jolla, California 92093

[3]Diversa Corporation
4955 Directors Place
San Diego, California 92121

[4]Institute of Biochemistry
Graz University of Technology
Petersgasse 12
A-8010 Graz
Austria

Summary

Bcl-2 family proteins regulate the release of proteins like cytochrome c from mitochondria during apoptosis. We used cell-free systems and ultimately a vesicular reconstitution from defined molecules to show that outer membrane permeabilization by Bcl-2 family proteins requires neither the mitochondrial matrix, the inner membrane, nor other proteins. Bid, or its BH3-domain peptide, activated monomeric Bax to produce membrane openings that allowed the passage of very large (2 megadalton) dextran molecules, explaining the translocation of large mitochondrial proteins during apoptosis. This process required cardiolipin and was inhibited by antiapoptotic Bcl-x_L. We conclude that mitochondrial protein release in apoptosis can be mediated by supramolecular openings in the outer mitochondrial membrane, promoted by BH3/Bax/lipid interaction and directly inhibited by Bcl-x_L.

Introduction

During apoptosis, Bcl-2 family proteins regulate the release of cytochrome c (Finucane et al., 1999; Jurgensmeier et al., 1998; Kluck et al., 1997; Luo et al., 1998; Yang et al., 1997) and other intermembrane space proteins, including Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000; O. von Ahsen and D.D. Newmeyer, submitted). The Bcl-2 family consists of proapoptotic members (e.g., Bid, Bax, and Bak), which trigger mitochondrial protein release, and antiapoptotic members (e.g., Bcl-2 and Bcl-x_L), which inhibit it. Some of the proapoptotic proteins have multiple Bcl-2-homology domains ("BH1-3" or "multidomain" proteins, including Bax and Bak) and some only one ("BH3-only" proteins, including Bid, Bim, Bad, and others). The BH1-3 proteins may be the primary effectors of apoptotic cell death, and the BH3-only proteins may generally act in concert with Bax or Bak (Lindsten et al., 2000; Wei et al., 2001; Zong et al., 2001).

How Bcl-2 family proteins regulate mitochondrial outer membrane permeability is controversial (Kelekar and Thompson, 1998). Several laboratories have attempted to reproduce this function using simplified artificial membrane systems (Basanez et al., 2001; Saito et al., 2000; Shimizu et al., 1999; Zhai et al., 2000, 2001). However, these systems have so far not been faithful models of mitochondrial membranes. On the other hand, studies with intact cells are complicated by the presence of multiple members of the Bcl-2 family and by the complexity of the cellular milieu.

To study individual Bcl-2-family proteins in a simple context while preserving their authentic functions, we used a stepwise reductionist approach, employing in vitro systems of decreasing complexity. Prior studies (Bossy-Wetzel et al., 1998; Kluck et al., 1997, 1999; Kuwana et al., 1998; von Ahsen et al., 2000) explored the release of mitochondrial proteins within whole cells and from isolated organelles. Here, we describe simplified systems consisting of isolated organellar membranes and liposomes that faithfully reproduced the behavior of whole mitochondria. Our data clarify the molecular requirements of the membrane permeabilization process and the functions of the Bcl-2 family.

Results

Apoptotic Function of Bid and Bax Proteins Reproduced with Mitochondrial Outer Membranes

We reconstituted the apoptotic process of protein release from mitochondria, and its control by Bcl-2 family proteins, using isolated outer mitochondrial membranes. Hypotonic treatment of the mitochondria separated many of the outer membranes from the mitoplasts, apparently intact (Figures 1A and 1B, asterisks). Isolated outer membranes had a vesicular structure with roughly the same diameter ($\sim$0.5 μm) and ellipsoidal shape as intact mitochondria (Figures 1C and 1D) and were referred to as outer membrane vesicles (OMVs). Control light membrane vesicles (LMVs) were smaller and more heterogeneous (Figures 1E and 1F). OMVs were enriched in outer membrane VDAC (Figure 1G) and virtually devoid of contamination with inner membranes (ANT), but contaminated to varying degrees with ER membranes (ribophorin). In contrast, LMVs contained only a trace of VDAC.

When the outer membrane isolation was performed in the presence of the protein GST, a proportion of the GST was retained in the final membrane fraction. Much of this was trapped in the lumen of the OMVs, as it was protected from protease digestion unless the membranes were solubilized by CHAPS (not shown). Thus, OMVs could entrap macromolecules.

Activities of Recombinant Proteins and Peptides on Mitochondria

To study the apoptotic permeabilization of outer mitochondrial membranes, we used protease-cleaved Bid

[5]Correspondence: don@liai.org

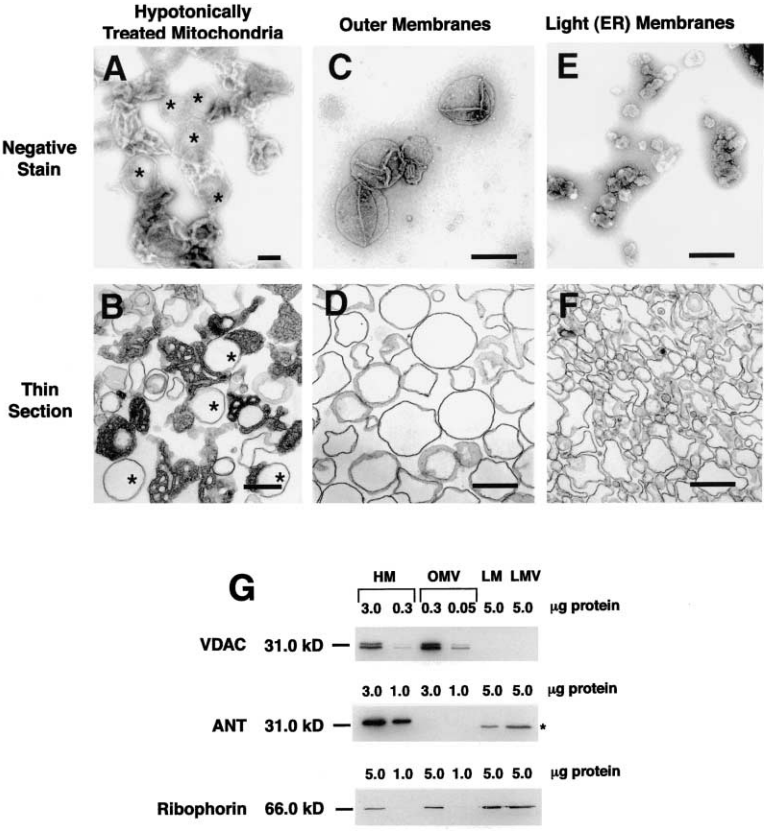

Figure 1. Outer Membranes Are Stripped off from the Mitoplasts in One Piece

(A) After the second hypotonic treatment, the mitochondrial suspension was negatively stained and examined by EM.

(B–D) Thin-section (80 nm) images of the same suspension. The electron-dense structures are mitoplasts or whole mitochondria, while the electron-transparent structures (asterisks) are outer membrane vesicles, seen more clearly in the purified outer membrane preparations ([C], negative stain; [D], thin section). Wrinkling in the OMVs in (C) is from flattening onto the grid.

(E and F) Negatively stained (E) and thin-sectioned (F) LMVs also displayed unilamellar profiles, but smaller. Bars represent 500 nm.

(G) OMVs are free of inner membrane-specific ANT. The purity of OMV and LMV preparations was analyzed by immunoblotting with antibodies to marker proteins: VDAC for the mitochondrial outer membrane, ANT for the inner membrane, and ribophorin for the ER membrane. HM and LM fractions were loaded on the same gel for comparison. VDAC was enriched in OMVs but undetectable in LMs and LMVs. ANT was absent from the OMVs. An apparently irrelevant crossreacting band (asterisk) seen in the LMs and LMVs migrates slightly faster than ANT.

(N/C-Bid; von Ahsen et al., 2000) and an activated oligomerized Bax (OG-Bax). Concentrations of 20–40 nM for N/C-Bid and OG-Bax were required for nearly complete cytochrome c release from *Xenopus* mitochondria in a 3 hr incubation (Figures 2A and 2C). Tumor cells typically contain 200–600 nM Bax (calculated from data of John Reed and colleagues, personal communication), and such high concentrations caused full cytochrome c release from *Xenopus* mitochondria within 10 min (not shown), as observed in apoptotic HeLa cells (Goldstein et al., 2000). N/C-Bid-induced cytochrome c release was blocked by low concentrations of Bcl-x_L (Figure 2B; 370 nM for the Bcl-$x_L\Delta C$ and 110 nM for the full-length protein). However, higher concentrations of Bcl-x_L ($\sim$10 μM) were required for inhibition of cytochrome c release induced by OG-Bax (Figure 2C).

Fluorescein-Dextran Release from OMVs and LMVs

When N/C-Bid and OG-Bax were added to OMVs previously loaded with fluorescein-dextrans (F-dextrans), the F-dextrans were released in a concentration-dependent manner (Figures 2D and 3B). Release was inhibited by Bcl-$x_L\Delta C$ and Bcl-x_L (Figure 2E) at the same concentrations effective with mitochondria (Figure 2B); a mutant Bcl-x_L protein (G138A) was ineffective (Figure 2F). LMVs did not release dextrans in response to Bid (Figure 2D) but did respond to OG-Bax, although less efficiently than OMVs (Figure 3D, far right).

Surprisingly, 10 and 2000 kDa dextrans (sizes confirmed by gel filtration; not shown) were released equally well (Figure 3). Release occurred without gross disruption of the membranes, as none of the N/C-Bid-permeabilized vesicles (Figures 4B, 4E, and 4G) differed in ultrastructure from the untreated OMVs (Figures 4A, 4D, and 4F). OG-Bax treatment also produced no observable membrane discontinuities (Figure 4C). A closer examination of en face images from thicker sections also showed that neither the control (GST-treated) nor the N/C-Bid-treated OMVs contained visible holes (Figures 4H and 4J). The efficient release of 2000 kDa dextrans in the absence of permanent disruptions in the lipid bilayer implies that Bid and Bax produced openings of supramolecular size in the outer membranes.

Dextran Release from Liposomes Formed from Extracted Organellar Lipids

To determine whether N/C-Bid or Bax alone could promote dextran release, we used protein-free liposomes (Figure 5A) prepared from extracted mitochondrial or microsomal lipids (M or ER liposomes, respectively). OG-Bax promoted the release of even large macromolecules (Figure 5B) when added at concentrations similar to those effective on intact mitochondria (Figure 2C) in the absence of any other proteins (including VDAC). Release from ER liposomes was less efficient than from M liposomes (Figure 5B), arguing that the lipid composition of mitochondrial membranes is important.

Neither N/C-Bid nor monomeric Bax added alone released dextrans from M or ER liposomes (Figures 5B and 5C). However, added together, these proteins promoted dextran release effectively from M, but not ER, lipo-

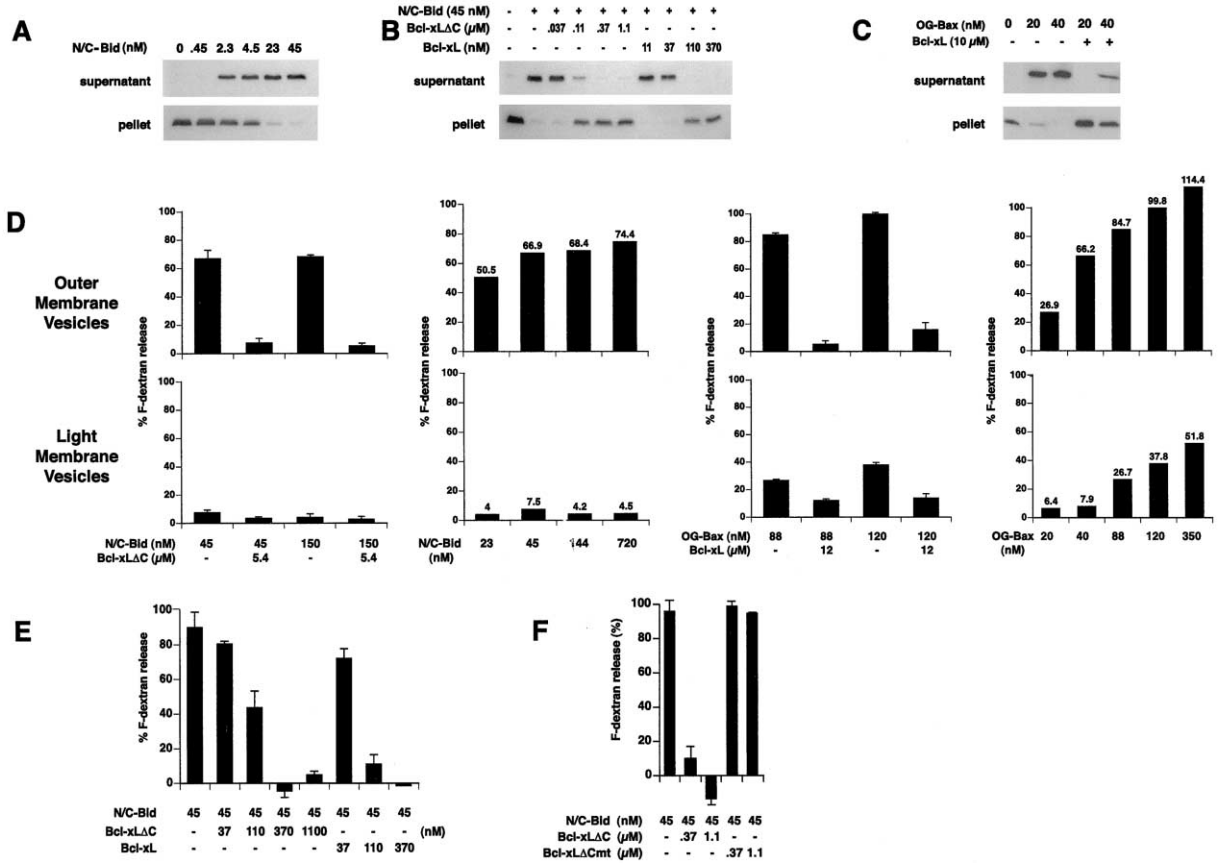

Figure 2. Outer Membrane Vesicles (OMVs) Reproduce Bax- and Bid-Induced Macromolecular Release

(A–C) Recombinant N/C-Bid and OG-Bax induced the complete release of cytochrome c from *Xenopus* mitochondria. Proteins were added at the indicated concentrations to isolated mitochondria in buffer. After an incubation for 3 hr at 22°C, cytochrome c release was detected by immunoblot analysis of the supernatant and pellet from each sample.

(D) N/C-Bid induced the release of fluorescein-dextran (10 kDa) from OMVs (top) and LMVs (bottom). N/C-Bid (left) released fluorescein-dextran in a concentration-dependent manner (see Figure 3 for a more detailed titration), and the release was blocked by Bcl-$x_L\Delta C$. Fluorescein-dextran-loaded LMVs did not respond to N/C-Bid (bottom). OG-Bax induced fluorescein-dextran (10 kDa) release (right) from OMVs (top), and the release was inhibited by full-length Bcl-x_L. LMVs (bottom) also responded to OG-Bax, but less efficiently than OMVs.

(E) Bid-induced dextran release from OMVs was inhibited by Bcl-$x_L\Delta C$ and Bcl-x_L at concentrations similar to those inhibiting cytochrome c release from mitochondria (B).

(F) A mutant Bcl-x_L (G138A) was unable to inhibit permeabilization. Values shown are mean ± standard deviation from triplicate data. Three other independent experiments gave similar results.

somes (Figures 5B and 5D). Hence, N/C-Bid cannot act alone to permeabilize membranes, but does so in combination with monomeric Bax. Moreover, lipid composition is critical. Importantly, even the very large 2 MDa dextran molecules were released from M liposomes (Figures 5C and 5D), and dextran release was inhibited by Bcl-x_L at the same concentrations active on whole mitochondria and OMVs. Thus, protein-free liposomes and Bcl-2 family proteins alone (Figure 5) can reproduce the macromolecular release properties of intact mitochondria and OMVs (Figures 2 and 3).

Although Bcl-x_L inhibited dextran release induced both by OG-Bax and by Bid/Bax synergy, the inhibition was much less complete with OG-Bax and indeed could be overcome by the addition of moderate concentrations of OG-Bax (Figure 5C). This suggests that the BH3-only, rather than the BH1-3, proteins may be the primary physiological target of Bcl-x_L and its antiapoptotic relatives, as others have argued (Cheng et al., 2001).

Dextran Release from Defined Liposomes: The Requirement for Cardiolipin

Figure 5B shows a marked effect of the source of extracted lipids. Whole mitochondria (inner and outer membranes combined) contained substantial levels of cardiolipin, essentially accounting for the difference between M and ER liposomes. Confirming this, we found that addition of cardiolipin to the ER lipids restored responsiveness of ER liposomes to Bid/Bax mixtures (not shown).

We formed liposomes with the lipid composition (excluding cholesterol) of *Xenopus* mitochondria, as determined by thin-layer chromatography: phosphatidylcholine, 46.5% ± 2.3%; phosphatidylethanolamine, 28.4% ± 3.5%; phosphatidylinositol, 8.9% ± 2.3%; phosphatidylserine, 8.9% ± 3.0%; and cardiolipin, 7.3% ± 0.6%. These defined liposomes responded well to a mixture of N/C-Bid and monomeric Bax, but not to either protein alone (Figures 6A–6D). Bcl-x_L blocked dextran release

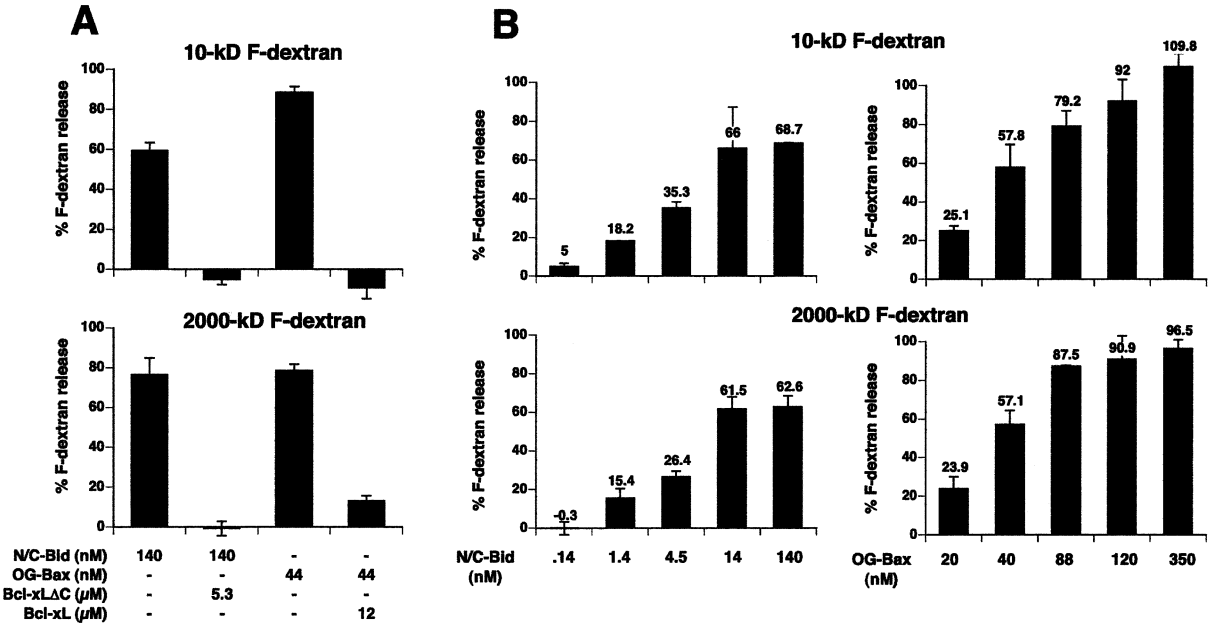

Figure 3. Small and Very Large Dextrans Are Released from OMVs by N/C-Bid or OG-Bax with the Same Efficiency

N/C-Bid or OG-Bax were added at the indicated concentrations to OMVs loaded with 10 kDa or 2000 kDa dextrans.

(A) During a 3 hr incubation, 10 and 2000 kDa fluorescein-dextrans were released from OMVs in response to N/C-Bid and OG-Bax, and in all cases the release was inhibited by Bcl-x_L.

(B) 10 and 2000 kDa dextrans were released equally by different concentrations of N/C-Bid and OG-Bax in a 3 hr time period. Values shown are the mean and range of duplicate data. Two other independent experiments gave similar results.

(Figure 6B) at concentrations similar to those effective with M liposomes, OMVs, and mitochondria; mutant Bcl-x_L (G138A) was inactive (Figure 6C). The response of liposomes to OG-Bax and Bid/Bax mixtures was highly dependent on levels of cardiolipin (Figure 6D), but did not require phosphatidylserine, another negatively charged lipid (not shown).

Activation of Bax by BH3 Peptide; Dependence on Cardiolipin

Synthetic BH3-domain peptides can induce cytochrome c release from isolated mitochondria (Cosulich et al., 1997; Polster et al., 2001). *Xenopus* mitochondria released cytochrome c, and OMVs released both 10 kDa and 2000 kDa dextrans, in response to wild-type Bid and Bad BH3 peptides at similar concentrations, but not to the mutant forms of the peptides (not shown); in both cases this release was inhibited by Bcl-x_L.

Importantly, a wild-type Bid BH3 peptide cooperated with monomeric Bax to induce dextran release from M but not ER liposomes (Figure 6E). Thus, N/C-Bid seems to act primarily through its BH3 domain. Moreover, membrane permeabilization is apparently accomplished by activated Bax, rather than by independent membrane interactions of both N/C-Bid and Bax. Again, Bcl-x_L inhibited this dextran release. Finally, Figure 6E suggests that cardiolipin was required even in the absence of the non-BH3 portions of Bid. Experiments with defined liposomes showed a similar cardiolipin dependence (not shown). Thus, cardiolipin is important not merely for the membrane targeting of Bid (Lutter et al., 2000), but also for the permeabilization function of Bax.

Massive CHAPS-Stable Bax Oligomerization Is Not Required for Permeabilization of M Liposomes

We analyzed Bax complexes by gel filtration (Figure 7A). Bax, when not treated with detergents such as octylglucoside, was essentially monomeric in solution, whether or not N/C-Bid (Figure 7A) or CHAPS (not shown) were added. However, in mitochondrial membranes, N/C-Bid shifted nearly all of the membrane-associated Bax to higher-order CHAPS-stable complexes, confirming previous reports (Antonsson et al., 2001). Similar results were obtained with OMVs, consistent with our other data showing that OMVs mimicked mitochondria in their response to N/C-Bid.

Surprisingly, however, massive N/C-Bid-induced Bax oligomerization was not seen in liposomes. On the other hand, the amount of ~100 kDa (tetramer-size) oligomers did correlate somewhat with permeabilization activity. In M liposomes, the addition of N/C-Bid produced a partial shift from the monomer to the ~100 kDa peak. In OMVs, Bcl-x_L caused Bax to shift almost entirely to monomer size; however, in M liposomes, only a partial shift toward the monomeric form was seen. These changes were less evident in ER liposomes. We conclude that massive CHAPS-stable Bax oligomerization is unnecessary for the permeabilization of M liposomes by Bid/Bax mixtures. Moreover, the Bax oligomers present in ER liposomes are insufficient for membrane per-

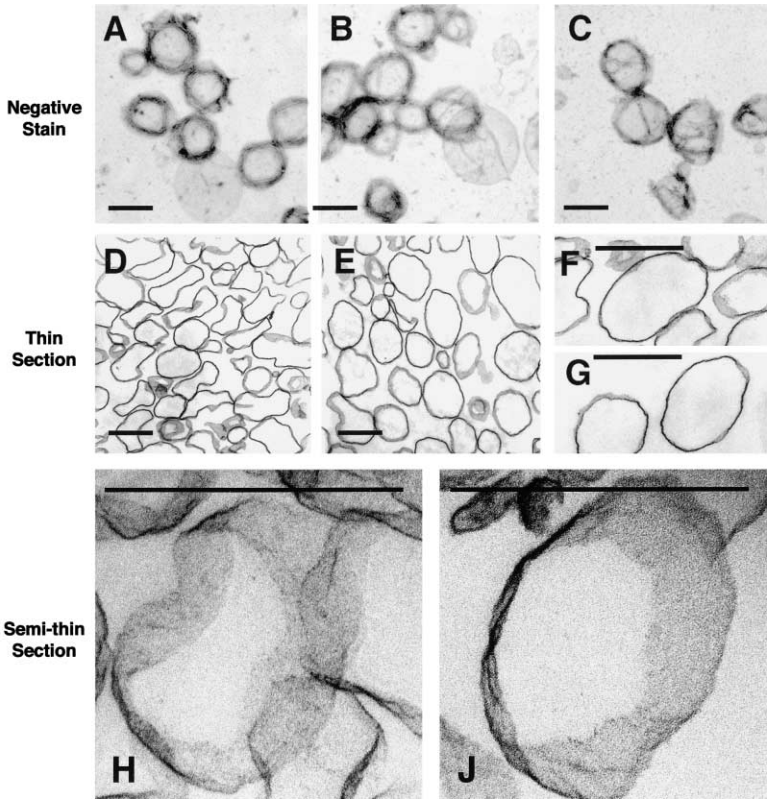

Figure 4. Bid and Bax Produce No Detectable Morphological Changes in OMVs, Despite Virtually Complete Release of 2000 kDa Dextrans

OMVs treated with N/C-Bid (B) or OG-Bax (C) were indistinguishable from control GST-treated OMVs (A) by negative-stain EM. Thin-section electron microscopy showed a similar lack of morphological changes in OMVs treated with GST (D and F) or N/C-Bid (E and G). Careful examination of en face images from thicker sections ([H], GST control; [J], N/C-Bid treated) revealed a lack of visible membrane discontinuities. Bars represent 500 nm.

meabilization. Finally, a modest correlation of the ~100 kDa peak with permeabilization suggests a role for tetramer-sized complexes (which also comprised a portion of the oligomers seen in mitochondria and OMVs).

The amount of Bax associated with M liposomes was unchanged in the presence of N/C-Bid or Bcl-x_L (Figure 7B). Moreover, similar amounts of Bax were recovered in M and ER liposomes (Figure 7A). Thus, although cardiolipin and N/C-Bid were essential for Bax-induced permeabilization, they were not required for the membrane association of Bax; moreover, Bcl-x_L blocked permeabilization without altering the binding of Bax to membranes.

Discussion

To investigate the molecular mechanisms of cytochrome c release from mitochondria in apoptosis, we developed cell-free systems that respond faithfully to pro- and antiapoptotic Bcl-2 family proteins under near-physiological conditions. Using resealed vesicles (OMVs) formed from mitochondrial outer membranes, we established a benchmark for judging the responsiveness of liposome-based systems. Dextran release from OMVs, like cytochrome c release from intact mitochondria (Kluck et al., 1997), was nearly complete, required similar concentrations of BH3 peptides or recombinant Bcl-2 family proteins, took place at physiological pH and temperature, and displayed a similar time course (not shown). Bax oligomerization in OMVs followed the same pattern as in whole mitochondria (Figure 7). Moreover, permeabilization was inhibited by the same concentrations of Bcl-x_L effective with whole mitochondria.

Our data show that both pro- and antiapoptotic Bcl-2 family proteins can regulate macromolecular efflux directly at the mitochondrial outer membrane. Bax formed supramolecular openings in mitochondrial outer membranes and liposomes, suggesting that the ion channel activity of Bcl-2 family proteins (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997, 1999) may be irrelevant for mitochondrial protein release in apoptosis. Furthermore, our results show that neither swelling of the mitochondrial matrix and inner membrane (Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1996; Vander Heiden et al., 1997; Zamzami et al., 1996) nor, indeed, any other process requiring ANT or the inner membrane is required for Bid/Bax-induced membrane permeabilization. Similarly, prior studies on cells and mitochondria concluded that cytochrome c release can take place in the absence of permeability transition, matrix swelling, or outer membrane rupture (Bossy-Wetzel et al., 1998; Eskes et al., 1998; Kluck et al., 1997, 1999; von Ahsen et al., 2000).

We conclude that permeabilization requires only the interaction of Bcl-2 family proteins such as Bax and Bid with the outer membrane. Other mitochondrial proteins, including VDAC, are not required for protein efflux. However, in principle other proteins could modulate the function or membrane localization of Bax, for example by altering lipid microdomains in the outer membrane or by modifying Bax postsynthetically.

Recent reports suggested that tBid by itself can permeabilize mitochondrial outer membranes (Grinberg et al., 2002; Kudla et al., 2000). However, N/C-Bid alone, even at concentrations (720 nM) well above those that activate monomeric Bax (45 nM), could not induce dex-

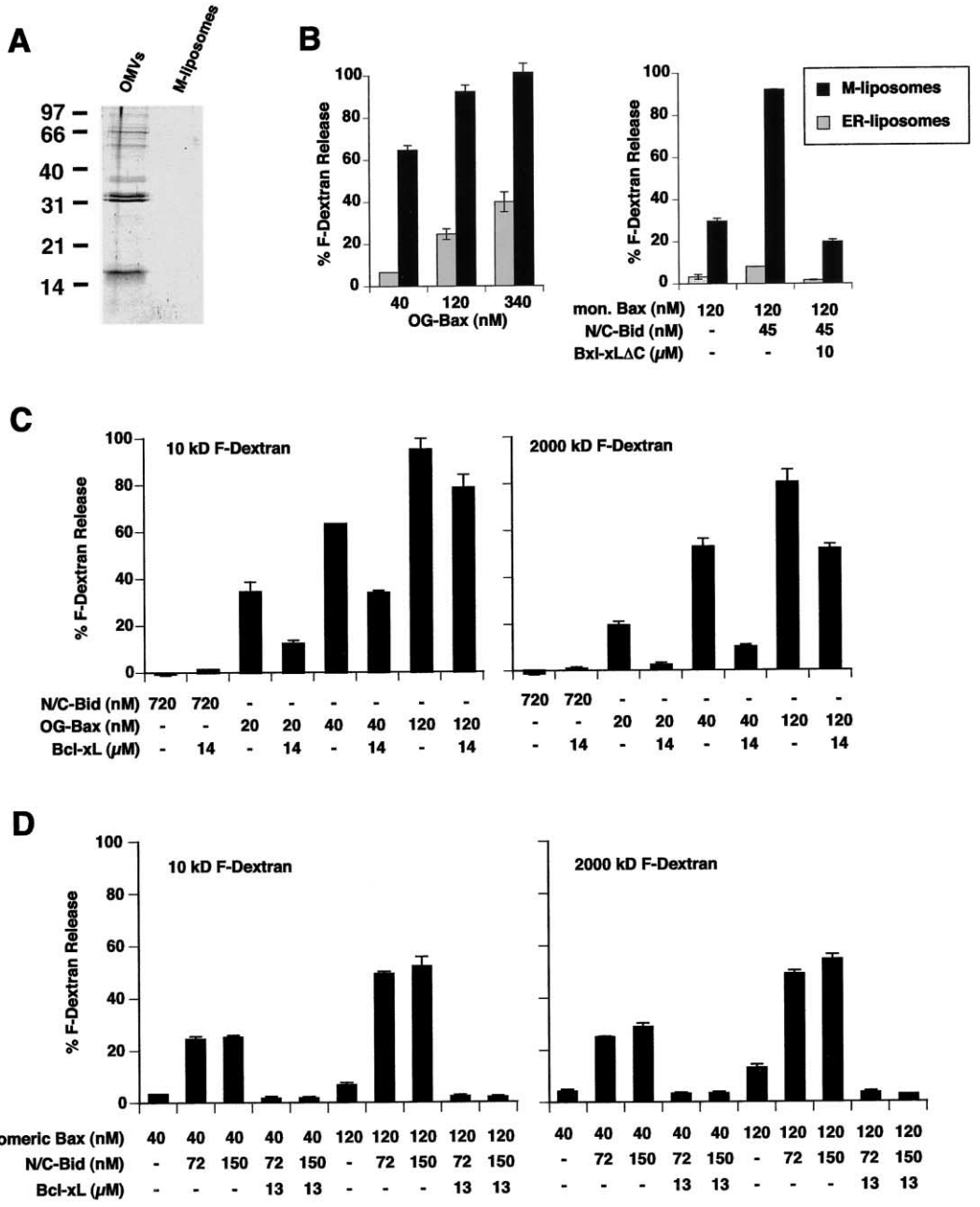

Figure 5. Liposomes Formed from Extracted Mitochondrial Lipids (M Liposomes) Release Dextrans in Response to OG-Bax and Mixtures of Monomeric Bax and N/C-Bid, whereas Liposomes Prepared from ER Lipids (ER Liposomes) Behave like LMVs

(A) Coomassie blue-stained SDS-12% polyacrylamide gel showing the near-absence of proteins in M liposomes (3000 μg total phospholipids loaded); the left lane shows OMVs equivalent to 105 μg of phospholipids.

(B) M and ER liposomes were treated with the indicated amounts of OG-Bax (left) or monomeric Bax, N/C-Bid, and Bcl-xLΔC (right), and dextran release measured as above. As with LMVs, the ER liposomes responded modestly, but reproducibly, to OG-Bax, but not to N/C-Bid or a mixture of monomeric Bax and N/C-Bid.

(C and D) M liposomes released both small and very large dextrans in response to OG-Bax (C) and to mixtures of N/C-Bid and monomeric Bax (D).

tran release from M liposomes (Figures 5B, 5C, 6B, and 6C); thus, Bid requires other proteins, such as Bax, to permeabilize membranes.

Whole mitochondria release proteins of various sizes in apoptosis. However, except in one recent report showing release of 10 and (less efficiently) 70 kDa dextrans by truncated Bcl-x_L (Basanez et al., 2001), permeability to macromolecules larger than cytochrome c has not been seen in artificial membrane systems. We now show, however, that Bid and Bax can efficiently release very large dextrans from liposomes and OMVs. This argues that no secondary event, beyond the initial ac-

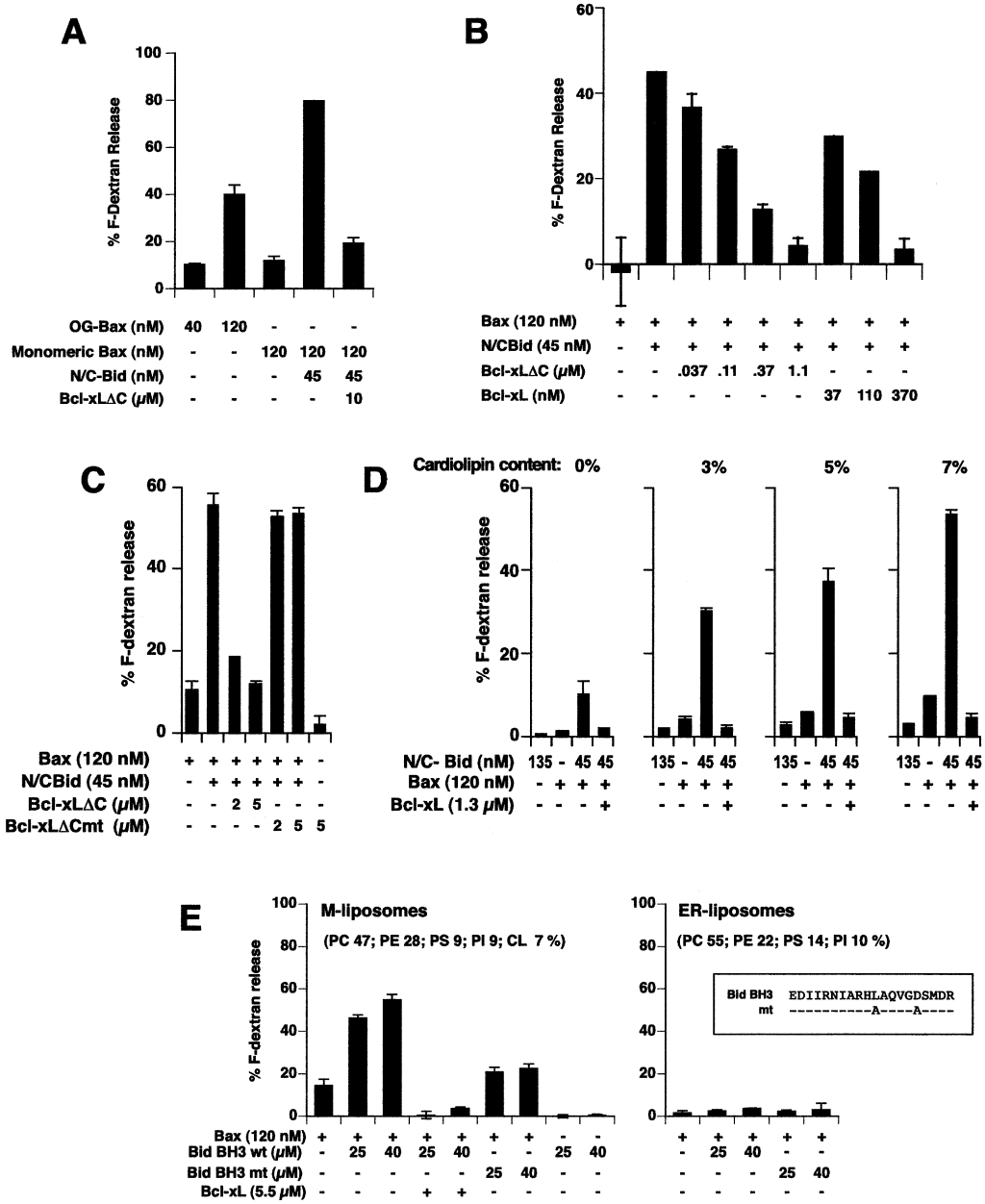

Figure 6. OG-Bax and Mixtures of Bax and N/C-Bid Permeabilize Synthetic Liposomes; Cardiolipin Is Required

(A) Liposomes formed from defined lipid mixtures similar to the observed composition of *Xenopus* egg mitochondrial membranes responded to OG-Bax or a mixture of N/C-Bid and monomeric Bax.

(B) Bcl-x_L and Bcl-$x_L\Delta C$ inhibit dextran release at the same concentrations effective in mitochondria, OMVs, and M liposomes (Figures 3B and 3E).

(C) A mutant Bcl-x_L (G138A) does not inhibit dextran release.

(D) Titration of cardiolipin. Except for cardiolipin, the lipid composition was as in (A).

(E) A Bid BH3 domain peptide can activate Bax to induce permeabilization of liposomes; cardiolipin is required. Dextran-loaded M and ER liposomes were treated with the indicated concentrations of wild-type and (less active) mutant Bid BH3 peptide, monomeric Bax, and Bcl-x_L. The lipid compositions of M and ER liposomes, estimated by thin-layer chromatography, are indicated at the top; note the similarity except for cardiolipin, which is specific for M liposomes.

tions of Bcl-2 family proteins such as Bid and Bax, is required for the release of larger proteins from the mitochondrial intermembrane space.

We have shown elsewhere that the membrane permeability caused by Bid or Bax in whole mitochondria can be reversed by the subsequent addition of Bcl-x_L (R.M. Kluck and D.D.N., unpublished data) and that Bid- or Bax-induced outer membrane openings allow only a limited rate of exchange of exogenous cytochrome c across the outer membrane (Kluck et al., 1999). Together with the present studies, these data argue that Bid and Bax can activate a subtle mechanism, independent of

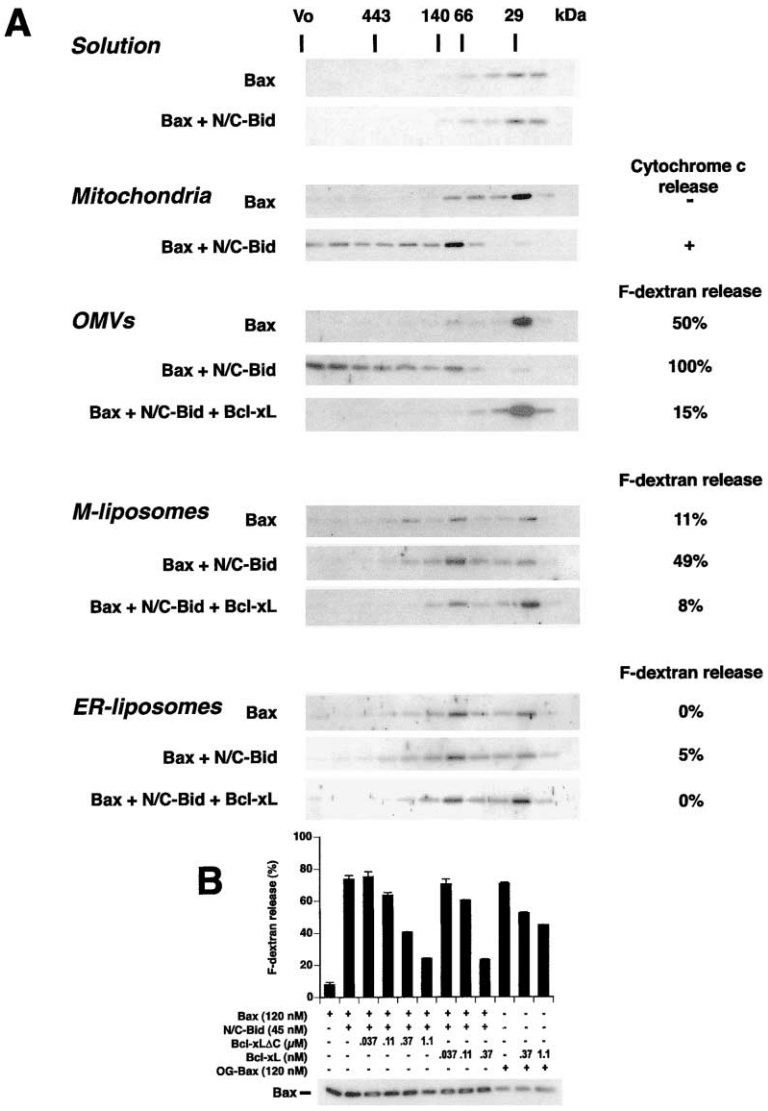

Figure 7. Massive CHAPS-Stable Bax Oligomerization and Increased Bax Association with Membranes Are Unnecessary for Membrane Permeabilization

(A) Analysis of Bax oligomerization. Bid induced massive CHAPS-stable Bax oligomerization in mitochondria and OMVs but not in solution; limited Bax oligomerization was seen in liposomes, but this was uncorrelated with membrane permeabilization. Bax was incubated alone, together with N/C-Bid, or with both N/C-Bid and Bcl-x_L, as indicated; incubations were done in buffer alone or in the presence of mitochondria, OMVs, M liposomes, or ER liposomes. Concentrations of recombinant proteins were increased in incubations with OMVs and liposomes, to compensate partially for the ~10-fold greater amount of membrane lipids. Protein concentrations were as follows: Mitochondria, 120 nM Bax and 45 nM N/C-Bid; OMVs, 400 nM Bax, 270 nM N/C-Bid, and 11 μM Bcl-x_L; and M and ER liposomes, 480 nM Bax, 270 nM N/C-Bid, and 11 μM Bcl-x_L. Following incubations, the membranes were collected by microfiltration, solubilized with 1.2% CHAPS, and subjected to gel filtration in the presence of 1.2% CHAPS.

(B) Bcl-x_L or N/C-Bid do not affect the association of Bax with M liposomes. The experiment was performed as in Figure 5B, except that after a 3 hr incubation with the recombinant proteins as indicated, membrane samples were reisolated and immunoblotted for Bax. Note also in (A) that the amount of Bax recovered from M and ER liposomes was similar; thus, cardiolipin does not influence Bax membrane association.

chemical energy and membrane potential, that permits large macromolecules to escape without permanently disrupting the lipid bilayer.

These results are inconsistent with a mechanism involving the formation by Bax of discrete protein channels (Saito et al., 2000; Shimizu et al., 1999). However, might the large-scale action of Bax involve massive oligomerization (Nechushtan et al., 2001)? Indeed, Bax oligomerization was induced by N/C-Bid in whole mitochondria and OMVs (Figure 7). Surprisingly, however, in liposomes only the smaller ~100 kDa complexes were correlated with membrane permeabilization. Thus, while massive CHAPS-stable Bax oligomers are unnecessary for membrane permeabilization, a certain threshold level of the ~100 kDa complexes may be required. It remains possible that larger oligomers form in liposomes, but are unstable or dissolved by CHAPS.

ER liposomes were not permeabilized by N/C-Bid and monomeric Bax (Figure 5B), arguing that mitochondrion-specific lipid composition is critical. The signature mitochondrial lipid is cardiolipin, which is present in inner membranes and probably also outer membranes (Ardail et al., 1990; de Kroon et al., 1997, 1999; Hovius et al., 1990; Lutter et al., 2000). Our data show that cardiolipin is important, not merely for the targeting of Bid (Lutter et al., 2000) or the membrane association of Bax, but for Bax function: the amount of Bax associated with M and ER liposomes was similar (Figure 7), despite the absence of cardiolipin in ER lipids, and cardiolipin increased dextran release induced by OG-Bax (Figures 5B and 7) and was required for release induced by mixtures of Bax and BH3 peptide (Figure 6E).

We hypothesize that activated Bax can promote dynamic localized alterations in the structure of the lipid bilayer, perhaps forming large transient lipidic pores or inverted lipid micelles (Hui et al., 1983; Kan, 1993; Rytomaa and Kinnunen, 1995; Siegel, 1984). Cardiolipin is known to exert "curvature stress" and can form inverted micelles (Siegel, 1984; Smaal et al., 1987), and thus could have a pivotal role in the physical process of membrane permeabilization.

The lipid dependence we observed also suggests a

potential mechanism for apoptotic regulation: the control of mitochondrial outer membrane permeabilization by molecules that affect the structure of the lipid bilayer, e.g., by altering the local concentration of cardiolipin or by otherwise modulating the ability of lipids to form altered structures in response to Bax or Bak. A recent report showed that tBid becomes localized near contact sites in mitochondrial membranes, which may therefore be enriched in cardiolipin (Lutter et al., 2001), in agreement with membrane subfractionation studies (Ardail et al., 1990; Simbeni et al., 1991). Although OMVs lack association with inner membranes, they may preserve such cardiolipin-rich domains.

A conundrum posed by our data is that the high cardiolipin content of the inner membrane should in theory permit Bax to permeabilize it; nevertheless, the inner membrane remains intact following Bid/Bax-induced cytochrome c release (Kluck et al., 1999; von Ahsen et al., 2000). Moreover, when N/C-Bid and Bax were added to mitoplasts (lacking intact outer membranes), mtHsp70, a soluble protein of the matrix, was not released (T.K. and D.D.N., unpublished). Indeed, this impermeability of inner membranes may be important for apoptotic cells to maintain, via oxidative phosphorylation, the ATP levels required for caspase activation. Interestingly, cardiolipin in the inner membrane may be restricted to the inner leaflet (Daum, 1985) and therefore inaccessible to Bax even after outer membrane permeabilization. In contrast, cardiolipin in the outer membrane may reside in the outer leaflet of the bilayer (Hovius et al., 1993), increasing the outer membrane's sensitivity to Bax.

Experimental Procedures

Organelle Fractionation

Xenopus egg extracts and mitochondrial (heavy membrane; HM) and light membrane (LM) fractions were prepared as described (Newmeyer et al., 1994; Newmeyer and Wilson, 1991; von Ahsen and Newmeyer, 2000).

Outer membrane vesicles (OMVs) from *Xenopus* egg mitochondria were isolated using an adaptation of the method developed for *N. crassa* mitochondria (Mayer et al., 1993, 1995), which were observed to be mostly right-side out. The HM fraction (200 μl) was diluted into 4 ml of HE buffer (5 mM HEPES/KOH [pH 7.4] containing 1 mM EDTA) for 10 min on ice. The lysate was pelleted at 5000 × g for 5 min, the supernatant was discarded, and the pellet resuspended in 2 ml of HE buffer (this additional hypotonic treatment of mitochondria increased the purity of the OMV preparation) containing 5 mg/ml fluorescein-dextran (10 kDa or 2000 kDa; Molecular Probes, D1821 and D7137) for 40 min on ice. LMs were resuspended in 10 pellet volumes of the same buffer with fluorescein-dextran and incubated on ice for 40 min. The organelle suspensions were homogenized with 30 strokes of a 2 ml Teflon-glass homogenizer on ice and loaded onto a sucrose step gradient consisting of 0.6 ml of 60%, 2.5 ml of 32%, and 1 ml of 15% sucrose in HE buffer. The gradient was centrifuged at 115,000 × g in an SW50 rotor (Beckman) for 1 hr at 4°C. The membrane layer at the interface between 32 and 15% sucrose was collected and adjusted to ~50% sucrose by the addition of 70% sucrose in buffer. This solution was overlaid with a flotation sucrose gradient consisting of 2 ml of 32% sucrose and 1 ml of HE buffer. After isopycnic centrifugation in an SW50 rotor at 115,000 × g for 16–18 hr at 4°C, the purified OMVs or LMVs were recovered at the interface between the 0% and 32% sucrose layers. The recovered membranes were diluted 5-fold in HE buffer and pelleted by centrifugation at 147,000 × g for 2 hr in an SW50 rotor and resuspended in HE buffer. Typical protein concentrations of the final OMV and LMV suspensions from the same pellet volume were ~0.5 and 2.0 mg/ml, respectively, by the BCA assay (Pierce), using BSA as a standard.

Recombinant Protein Production

Cleaved human Bid (N/C-Bid) was produced using the pGEX-4T-1 vector; a thrombin cleavage site was introduced in place of the caspase cleavage site in the Bid molecule, and six histidines were inserted at the C terminus (von Ahsen et al., 2000). Protein production was induced for 4 hr with 1 mM IPTG in BL21(DE3) cells (Novagen) at 37°C, which were lysed without detergent by sonication. The protein was applied to glutathione-Sepharose (Amersham Pharmacia Biotech), eluted with 20 mM glutathione in 50 mM Tris/HCl (pH 8.0), and digested with thrombin (Amersham; 120 U/ml, 22°C overnight). In this way, Bid was cleaved internally and from GST at the same time; the two fragments of the molecule remained associated (Chou et al., 1999; not shown). N/C-Bid was then applied to Ni-NTA resin (Qiagen) and eluted with 250 mM imidazole; the contaminating GST was cleared with glutathione-Sepharose. GST protein was expressed similarly from BL21(DE3) harboring pGEX-4T-1 (Amersham), eluted from glutathione-Sepharose, and further purified on Mono Q (Amersham).

Full-length human Bax protein was produced as described (Suzuki et al., 2000). For OG-Bax, Bax was oligomerized by the addition of 0.7% octylglucoside. The final concentration of octylglucoside in the assay was only 0.035%, and this amount of detergent alone did not produce a detectable release of fluorescein-dextran. Human Bcl-$x_L\Delta C$ was prepared as described (Muchmore et al., 1996) and further purified on Mono Q. Full-length human Bcl-x_L was produced as a GST-fusion cloned in pGEX 4T-1. BL21(DE3) cells were transformed and grown in 2YT to $OD_{600} = 0.7$, and protein expression was induced with 1 mM IPTG at 25°C for 16–18 hr. Cells were lysed by sonication, and the supernatant was incubated with glutathione-Sepharose for 1.5 hr at 22°C. The Bcl-x_L protein was cleaved off GST on the beads using thrombin (50 U/ml, Amersham) for 18 hr at 22°C and purified on Mono Q; the peak fraction was dialyzed in HE buffer. These proteins were >90% pure electrophoretically.

Gel Filtration of Bax and Bax/Bid

Bax (0.15 μg) and N/C-Bid (.005 μg) were mixed and incubated for 1.5 hr at room temperature, and then this mixture (or Bax alone) was loaded onto a Superdex 200HR 10/30 column (Amersham) and eluted at 0.3 ml/min in 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.2 mM DTT, ±1.2% CHAPS. Fractions were used for Bax immunoblot or the dextran release assay. With membrane-associated Bax, membrane fractions were isolated with a microfiltration unit (0.1 μm pores) and solubilized with 100 μl of elution buffer containing 1.2% CHAPS prior to chromatography.

Immunoblot Analysis

Samples were subjected to SDS-PAGE (12% or 15% gels) and transferred to nitrocellulose (BioRad, 0.45 μm). The membranes were probed with antibodies to VDAC (1:5000; Calbiochem, 31HL; mitochondrial outer membrane), ANT (1:500; a gift from P. Schmid, University of Minnesota, USA; inner membrane), or ribophorin (1:200; a gift from E.B. Lane, University of Dundee, UK; ER membrane). Cytochrome c and Bax were detected with antibodies 65981A (1:3000; Pharmingen) and N20 (1:1000; Santa Cruz), respectively. After a secondary reaction with horseradish peroxidase-conjugated anti-mouse- or anti-rabbit-Ig antibodies (1:2000; Amersham), the membranes were incubated with ECL reagent (Amersham) or (for Bax) Supersignal West Femto (Pierce) and exposed to the film for 10 s to 1 min.

Dextran Release Assay

LMVs or OMVs (2.5 μl) were used in a 50 μl assay mixture in 30% phosphate-buffered saline with recombinant proteins and/or BH3 domain peptides and incubated at 22°C for 3 hr. Carrier protein (GST, 75 μg/ml) was added to all samples to block nonspecific losses. After incubation, the released fluorescein-dextran was collected through either a 100 kDa microfiltration unit (Amicon) or 0.1 μm pore-size filters (Millipore) and detected with a spectrofluorometer (Photon Technology International) with excitation at 490 nm and emission at 520 nm. The baseline was taken as the fluorescence

obtained with the addition of GST only (75 μg/ml) and 100% release as that obtained after 1% CHAPS solubilization. The efficiency of permeabilization depended only weakly on the protein/lipid ratio (not shown).

Cytochrome c Release Assay with *Xenopus* Mitochondria

HMs (4 μl) were added to a 50 μl assay mix consisting of 40 μl of ELB with 120 mM KCl and 1 μl of BH3-domain peptides from a stock in DMSO or 10 μl of recombinant proteins. After 3 hr at room temperature, 20 μl of the sample was spun at 20,000 × g for 5 min; 10 μl of the supernatant was collected for immunoblot analysis. The pellet was resuspended in 20 μl of sample buffer and 10 μl of this was analyzed by immunoblot with anti-cytochrome c antibody.

Electron Microscopy (EM)

Negative Stain

1–4 μl of the final vesicle suspension was applied onto a formvar-covered and carbon-coated 300 mesh grid. After 30 s, excess fluid was removed with filter paper. The sample was immediately stained with 0.5%–1% uranyl acetate for 5–30 s. Excess fluid was again removed with filter paper and the grids were examined in either a JEOL 1200 or a JEOL 2000 FX electron microscope (Nihondenshi, Japan) at 80kV.

Transmission

After the final high-speed centrifugation of the OMV or LMV preparations, the pellet was fixed with 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) for 2 hr. Hypotonically treated mitochondria were pelleted at 100,000 × g for 15 min and fixed in the same way. Subsequent processing was as described (Ghoshroy et al., 1995).

For a more detailed observation of en face views of OMVs, both Bid- and control GST-treated OMV samples were sectioned at 100–120 nm. Sections were mounted on 300 mesh copper grids and poststained with 1% uranyl acetate and Sato lead. OMVs were examined with a JEOL 2000 FX electron microscope at 80kV and tilted at 40°–45° for an en face view. Vesicles were randomly selected in a section, and images were subsequently recorded using a Tietz TemCam-F224 2K × 2K CCD camera. Thirty to forty images were examined for the control and Bid-treated vesicles.

Formation of Liposomes from Extracted Organellar Lipids

HMs or LMs (0.5 ml) were mixed with 3 ml of chloroform/methanol (1:1) and vortexed eight times for 30 s each. After addition of 1 ml of water, the mixture was vortexed again six times and centrifuged at 500 × g for 10 min. The bottom layer was collected by Pasteur pipette and transferred to a glass bottle on ice. An additional 2 ml of chloroform was added to the remaining sample, which was then vortexed four times; the bottom layer was pooled with the first extract. The extract was dried under a nitrogen stream for 3–5 hr and sometimes stored at −20°C. The lipid film was hydrated in 1 ml of potassium phosphate buffer (5 mM potassium phosphate [pH 7.4], 50 mM KCl, and 1 mM EDTA) containing 5 mg/ml fluorescein-dextran (10 kDa or 2000 kDa) and placed in a water bath sonicator (Branson 2510) for 5 min. The suspension was passed 31 times through a 400 nm pore size filter membrane (Corning) fitted in an extruder (Avestin). The sample (0.5 ml) was mixed with 1 ml of 50% sucrose in potassium phosphate buffer, loaded at the bottom of a centrifuge tube, and overlaid by 1 ml each of 15%, 10%, and 0% sucrose. The gradients were centrifuged at 115,000 × g for 19–20 hr in an SW-50 rotor (Beckman). The layer from the top to the interface between 0% and 10% sucrose was collected and washed in potassium phosphate buffer at 147,000 × g for 2 hr. The pellet was resuspended in the same buffer. The volume of the buffer was adjusted to the size of the pellet in the same way as for the OMV preparation.

Lipid Class Composition Analysis

Lipid extracts of subcellular membrane fractions (Bligh and Dyer, 1959) were dried under a stream of nitrogen and dissolved in 50 μl of methanol/chloroform (2:1). Phospholipid classes were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% ammonia (65:35:5, per vol.) as the first and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, per vol.) as the second developing solvent. Spots detected after exposure to iodine vapor were scraped off. Total and individual phospholipids were quantified as described (Broekhuyse, 1968).

Formation of Liposomes from Defined Lipid Mixtures

Egg phosphatidylcholine (PC), egg phosphatidylethanoloamine (PE), brain phosphatidylserine (PS), soybean phosphatidylinositol (PI), and heart cardiolipin (CL) in chloroform (Avanti Polar Lipids) were mixed in a glass bottle at the molar ratios indicated in the text. Lipids were dried under nitrogen, resuspended in potassium phosphate buffer (pH 7.4) at 8 mg/ml, and processed as described for M and ER liposomes.

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