ATP depletion results in Bax translocation from cytosol to mitochondria and release of cytochrome c from mitochondria into cytosol in cultured kidney cells. Overexpression of Bcl-2 prevents cytochrome c release, without ameliorating ATP depletion or Bax translocation, with little or no association between Bcl-2 and Bax as demonstrated by immunoprecipitation (Saikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., Weinberg, J. M., and Venkatachalam, M. A. (1998) Oncogene 17, 3401–3415). Now we show that translocated Bax forms homo- or oligomeric structures, stabilized as chemical adducts by bifunctional cross-linkers in ATP-depleted wild type cells, but remains monomeric in Bcl-2-overexpressing cells. The protective effects of Bcl-2 did not require Bcl-2/Bax association, at least to a degree of proximity or affinity that was stable to conditions of immunoprecipitation or adduct formation by eight cross-linkers of diverse spacer lengths and chemical reactivities. On the other hand, nonionic detergents readily induced homodimers and heterodimers of Bax and Bcl-2. Moreover, associations between translocated Bax and the voltage-dependent anion channel protein or the adenine nucleotide translocator protein could not be demonstrated by immunoprecipitation of Bax, or by using bifunctional cross-linkers. Our data suggest that the in vivo actions of Bax are at least in part dependent on the formation of homo- or oligomers without requiring associations with other molecules and that Bcl-2 cytoprotection involves mechanisms that prevent Bax oligomerization.

Mitochondria are central to the apoptosis activation pathway in many physiological and pathological conditions. Members of the Bcl-2 family of proteins are known to affect mitochondrial function and regulate the release of apoptosis-activating factors (2–5). Anti-apoptotic members of Bcl-2 family (e.g. Bcl-2 and Bcl-xL) act primarily to preserve mitochondrial integrity by suppressing the release of cytochrome c (5). In contrast, pro-apoptotic members (Bax, Bid, etc.) induce the release of cytochrome c and cause mitochondrial dysfunction (1, 6–8). The pro-apoptotic protein, Bax, which normally resides in the cytosol, translocates to mitochondria when triggered by certain stimuli (6, 9). Translocated Bax has been shown to induce cytochrome c release both in vivo (1, 6, 10) and in vitro (11) and this is followed by caspase activation (10, 12). The mitochondrial permeability transition, an event that results in disruption of the mitochondrial potential gradient, has been reported to induce cytochrome c release and apoptosis (13). However, our observations and several other reports suggest that the effects of Bax are targeted at the outer mitochondrial membrane and that the mitochondrial inner membrane remains intact even after Bax-induced release of cytochrome c (6, 10, 14–16).

How cytochrome c leaves mitochondria during apoptosis after relocation of Bax to the mitochondrial outer membrane still remains an unanswered puzzle. Potential mechanisms involve mitochondrial swelling caused by opening the permeability transition pore in the inner membrane (17) or by mitochondrial hyperpolarization followed by swelling and membrane rupture (18). However, it has been reported that the pro-apoptotic proteins Bid and Bax can release cytochrome c from isolated mitochondria in the absence of detectable mitochondrial swelling (19). Although it was believed earlier that Bax induces the release of cytochrome c by inhibiting Bcl-2 function through binding of the Bcl-2 homology domains BH1, BH2, and BH3, there is evidence to suggest that Bax and Bcl-2 function independently in regulating apoptosis (20, 21). Formation of ion channels in synthetic lipid bilayer by members of the Bcl-2 family (22) has suggested that pro-apoptotic members may reaggregate in the outer mitochondrial membranes to allow the efflux of cytochrome c by forming large channels. Even though both Bcl-2 and Bax are capable of forming ion channels in artificial membranes, it is unclear how these proteins can form similar channels and still exert opposing actions. The data would suggest that Bax function can be inhibited by Bcl-2/Bcl-xL, but does not require direct Bax/Bcl-2 or Bax/Bcl-xL interaction for regulating Bax function (23, 24). For example, enforced dimerization of Bax, as a chimeric protein with FK506 binding protein, resulted in its translocation to the mitochondria and induced cell death even in the presence of Bcl-xL (25). Likewise, Bax mutant proteins that fail to bind to Bcl-2 are capable of inducing apoptosis (20). In addition, Youle’s group (26) have shown that nonionic detergents induce Bax homo- and heterodimerization with Bcl-2 or Bcl-xL and suggested that such simple dimers alone are not sufficient to regulate apoptosis. Bax has recently been reported to interact directly with VDAC3.

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on the outer membrane to release cytochrome c or with ANT on the inner membrane to initiate the permeability transition, indirectly leading to cytochrome c release (27, 28). More recently, it has been reported that Bax may cause instability in artificial lipid membranes, suggesting another mechanism by which Bax may permeabilize the outer mitochondrial membrane (29). A caveat in this model is that Bcl-xL, which is known to block cytochrome c release from intact mitochondria, did not prevent the membrane-destabilizing effects of Bax.

We have been investigating the roles played by Bax and Bcl-2 in the regulation of cytochrome c release from mitochondria in a model of apoptotic cell death induced by cellular ATP depletion. We have shown previously that severe ATP depletion of cultured rat kidney proximal tubule cells induced by hypoxia or chemical inhibitors of mitochondrial respiration triggers the translocation of cytosolic Bax to mitochondria and cytochrome c release into the cytosol (1). In this model, mitochondrial insertion of Bax does not compromise the integrity of inner mitochondrial membranes (14). Thus, the outer mitochondrial membrane would appear to be a reasonable site for the permeabilizing actions of Bax, at least in the context of hypoxia. Using this model, we now report that, following insertion into the mitochondrial outer membrane, Bax oligomerizes to form a multimeric structure that could explain the release of cytochrome c. We also show that the protective actions of Bcl-2 may stem from its ability to block Bax oligomerization in the mitochondrial outer membrane without forming physical complexes with the Bax protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

Materials purchased from vendors were as follows. Ham’s F-12/Dulbecco's modified Eagle’s medium were from Life Technologies, Inc. Monoclonal antibodies to rat Bax (ID1) were kindly provided by Dr. Richard J. Youle (National Institutes of Health, Bethesda, MD) and polyclonal antibody to rat adenine nucleotide translocator was provided by Dr. H. H. Schmid (Hormel Institute, Austin, MN). Anti-cytochrome c monoclonal antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA); anti-Bcl-2 polyclonal antibody (AC 21) and anti-Bax polyclonal antibody (P-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-porin 31 HL (α-VDAC) monoclonal antibodies Ab-1, Ab-2, Ab-3, and Ab-4 were from Calbiochem (San Diego, CA). Horse-radish peroxidase-conjugated and preadsorbed secondary antibodies to mouse and rabbit were obtained from Jackson Immunoresearch Laboratories (Westgrove, PA). Rat kidney proximal tubule cells (RPTC; Dr. H. H. Schmid (Hormel Institute, Austin, MN). Anti-cytochrome c monoclonal antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA); anti-Bcl-2 polyclonal antibody (AC 21) and anti-Bax polyclonal antibody (P-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-porin 31 HL (α-VDAC) monoclonal antibodies Ab-1, Ab-2, Ab-3, and Ab-4 were from Calbiochem (San Diego, CA). Horse-radish peroxidase-conjugated and preadsorbed secondary antibodies to mouse and rabbit were obtained from Jackson Immunoresearch Laboratories (Westgrove, PA). Rat kidney proximal tubule cells (RPTC; Dr. H. H. Schmid (Hormel Institute, Austin, MN). Anti-cytochrome c monoclonal antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA); anti-Bcl-2 polyclonal antibody (AC 21) and anti-Bax polyclonal antibody (P-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-porin 31 HL (α-VDAC) monoclonal antibodies Ab-1, Ab-2, Ab-3, and Ab-4 were from Calbiochem (San Diego, CA). Horse-radish peroxidase-conjugated and preadsorbed secondary antibodies to mouse and rabbit were obtained from Jackson Immunoresearch Laboratories (Westgrove, PA). Rat kidney proximal tubule cells (RPTC; Dr. H. H. Schmid (Hormel Institute, Austin, MN). Anti-cytochrome c monoclonal antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA); anti-Bcl-2 polyclonal antibody (AC 21) and anti-Bax polyclonal antibody (P-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-porin 31 HL (α-VDAC) monoclonal antibodies Ab-1, Ab-2, Ab-3, and Ab-4 were from Calbiochem (San Diego, CA). Horse-radish peroxidase-conjugated and preadsorbed secondary antibodies to mouse and rabbit were obtained from Jackson Immunoresearch Laboratories (Westgrove, PA).

**ATP Depletion by CCCP**

Cells were cultured in serum-supplemented Ham’s F-12/Dulbecco’s modified Eagle’s medium with 17.5 mM glucose as described (30) and plated at 10^6 cells/cm^2 in 60- or 100-mm collagen-coated dishes. After overnight growth, cells were washed with phosphate-buffered saline and subjected to ATP depletion by incubation in glucose-free Krebs-Ringer bicarbonate buffer (in mM: 115 NaCl, 1 KH2PO4, 4 KCl, 1 MgSO4, 1.25 CaCl2, and 25 NaHCO3; pre-gassed with 95% N2 air, and 5% CO2) containing 15 μM CCCP at 37 °C under normoxic conditions. Glycine (0.8 μM) was included in the buffer to stimulate glycine contents of tissues in vitro (31, 32), thus preventing early necrotic injury during incubation (33).

**Preparation of Subcellular Fractions**

**Protocol I—Cytosolic and membrane fractions were prepared by selective plasma membrane permeabilization with digitonin (34), followed by membrane solubilization. Briefly, control and experimental cells in dishes were treated with 0.05% digitonin in isotonic buffer A (10 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, pH 7.4; ~ 10^6 cells/ml) containing protease inhibitors (1 mM 4-(2-aminophenyl)benzenesulfonyl fluoride hydrochloride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A), for 1–2 min at room temperature. Cell permeabilization by digitonin was standardized by measuring 100% release of lactate dehydrogenase and was also monitored visually under an inverted microscope. The permeabilized cells were shifted to 4 °C, scraped with a rubber policeman, and collected into centrifuge tubes. The supernatants (Dig/Cytosol) were routinely collected after centrifugation at 15,000 × g for 10 min. Following centrifugation, the pellet was further extracted with ice-cold detergent (1% Nonidet P-40 or Triton X-100 or CHAPS) in buffer A containing protease inhibitors for 60 min at 4 °C to release membrane- and organelle-bound proteins including mitochondrial cytochrome c. Both detergent-soluble (membrane) and insoluble fractions were collected by low speed (15,000 × g) or high speed (500,000 × g) centrifugation. The protein patterns of soluble membrane fractions, after low or high speed centrifugation, both by SDS-PAGE and Western blotting were indistinguishable. Therefore, solubilized membrane fractions were routinely collected by centrifugation at 15,000 × g for 10 min. The relative protein levels of Dig/Cytosol, membrane- and detergent-insoluble fractions are 58 ± 4%, 15 ± 0.5%, 27 ± 4% for control, and 48 ± 6%, 14 ± 0.5%, 38 ± 6% for ATP-depleted cells (4 h CCCP), respectively (data from four independent experiments).

**Protocol II—Subcellular fractionation of cells was also achieved by Dounce homogenization in isotonic Buffer B (250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, pH 7.4) and differential centrifugation yielding nuclear (500 × g × 10 min), mitochondrial (15,000 × g × 1 min), microsomal (500,000 × g × 1 min) and cytosol (500,000 × g × 10 min) supernatant fractions. Unbroken cells constituted ~1%, as monitored by light microscopy with trypan blue. Unlike control cells, ATP-depleted cells contained large numbers of altered mitochondria that sedimented with nuclei. In order to make valid comparisons, nuclear and mitochondrial fractions were collected together as 15,000 × g pellet. Protein concentration was estimated with bicinchoninic acid (BCA) reagent (Pierce) following supplier’s protocol using bovine serum albumin as standard.**

**Protein Cross-linking**

All cross-linkers were dissolved in Me2SO just before using. Cross-linkers were added at 1 mM (0.1 mM for SANPAH) concentration to intact cells (~2 × 10^6 cells equivalent to 1 mg of total protein), cells permeabilized with digitonin or detergent extracts of membrane. Optimum cross-linking conditions for cytosol and membrane extracts were determined after testing different cross-linker to protein ratios. 1 mM concentration of all cross-linkers except for SANPAH (0.1 mM) was found to be optimal for a range of protein concentrations (100–500 μg) in the extracts. After the addition of cross-linkers, cells or extracts were incubated on a head-to-head rocker for 30 min at room temperature. Amine targeting cross-linkers (NHS esters and imido esters) were quenched by adding 0.1 volume of 2 M Tris-HCl (pH 7.4) and incubated with rocking for another 30 min at room temperature. Sulphydryl targeting cross-linker DPDPB was removed from cells by washing the pellets or from extracts by protein precipitation with 3% trichloroacetic acid or 80% acetone. In case of SANPAH, all the incubations were carried at 4 °C with 20 min of incubation to react NHS esters and 10 min of exposure to UV light (360 nm) to generate the nonspecifically reactive nitrenes. After cross-linking, cytosol and membrane fractions from intact cells were collected as described above. Cleavage of S-S-bridge-containing cross-linkers was achieved by incubating extracts with 50 mM DTT for 30 min at 37 °C.

**Immunolocalization**

Proteins were resolved by non-reducing or reducing (50 mM DTT) SDS-PAGE, blotted onto Xcell II mini gel on 10% or 4–12% (gradient) NuPAGE gels (Invitrogen, CA) using MES or MOPS running buffer, as recommended by the manufacturer. After electrophoresis, proteins from the gel were electroblotted onto 0.2-μm PVDF membranes following manufacturer’s directions. Western blotting using appropriate primary antibodies and peroxidase-conjugated suitable secondary antibodies was performed to analyze proteins. Chemiluminescent substrates (Pierce) were used to detect antigen-antibody complexes on the PVDF membrane.
centrifugation as described under “Experimental Procedures.” Insoluble matrix fraction was dissolved in SDS/sample buffer, and all the fractions were applied on top of the gel. The gel was focused for 1 h at 100 V, 3 h at 500 V, and 0.5 h at 500 V using anode (7 mM phosphoric acid) and cathode (20 mM arginine, 20 mM lysine) buffers at room temperature. Proteins in IEF gels were electroblotted onto a 0.2-μm PVDF membrane for 1 h in 0.7% (v/v) acetic acid, pH 3.0, and were detected by appropriate antibodies. The pH gradient on the IEF gel was determined either by surface electrode or by pH measurement of deionized water eluate of focused gel slices (0.5-cm width).

RESULTS

Relative Distribution of Bax in Normal and ATP-depleted Cells—We have shown previously that cultured rat kidney proximal tubule cells express high concentrations of Bax, which is localized in the cytosol (1). ATP depletion by either hypoxia or treatment with CCCP in the absence of glucose causes Bax translocation to mitochondria and cytochrome c release into cytosol (1). Provision of growth medium after hypoxia or chemically induced ATP depletion allows resynthesis of ATP by glycolysis and causes apoptotic death in cells with cytosolic cytochrome c (1). As shown in Fig. 1A, Bax is predominantly cytosolic in control cells (>99% soluble) and migrates to membranes (mitochondria) in ATP-depleted cells (>90% membrane-bound; Fig. 1A, lanes 2 and 3). The relative percentages of soluble and membrane-bound Bax in control and ATP-depleted RPTC were determined by densitometric analysis of chemiluminescence signals on films after Western blotting. From several experiments, we found that, after 4 h of ATP depletion, the
depleted cells (Fig. 1) in normal cells but co-localized to the same fractions in ATP-translocates exclusively to the mitochondria (1). In accordance with cytochrome oxidase, a mitochondrial marker, re-centrifugation. These results further confirmed the soluble nature of Bax protein in normal cells and validated the use of digitonin to obtain cytosol from whole cells.

Double immunostaining, using an anti-Bax antibody and an antibody to cytochrome oxidase, a mitochondrial marker, revealed that the localization of Bax coincided exactly with that of cytochrome oxidase during ATP depletion, showing that Bax translocates exclusively to the mitochondria (1). In accordance with our earlier results, Bax and cytochrome oxidase were shown in the current study to be present in different fractions in normal cells but co-localized to the same fractions in ATP-depleted cells (Fig. 1B, lanes 2 and 3). In order to assess Bax translocation by conventional cell fractionation, Dounce homogenization followed by differential centrifugation was carried out. Control cells provided clean fractions of nuclei, mitochondria, microsomes, and cytosol. Integrity and specificity of mitochondrial fractions were confirmed by the presence cytochrome c (data not shown). Interestingly, although mitochondria appeared filamentous in normal cells, they were round and aggregated around the nucleus in ATP-depleted cells (1), suggesting that ATP depletion has changed the shape and densities of mitochondria. The modified shape and densities of mitochondria posed a challenge in obtaining clean mitochondrial fractions in ATP-depleted cells. Therefore, mitochondria and nuclei were collected together to assess Bax translocation. Together with our previous immunocytochemical observations showing that nuclei of ATP-depleted cells are devoid of Bax and that the protein is visualized exclusively in mitochondria (1), these fractionation studies provided good evidence that Bax translocates to mitochondria, but not nuclei or the microsomal membranes during ATP depletion (Fig. 1B, lanes 4–6). Based on these results, we routinely used the crude membrane fractions that remained after the removal of digitonin-released cytosol to assess Bax localization. Separate studies showed that membrane bound Bax was efficiently extracted with detergents, and remained in the supernatants to the same extent regardless of whether the extracts were centrifuged at 15,000 × g or 500,000 × g. Thus assay of detergent extracts of the crude membrane fraction represented a valid method to assess Bax in mitochondrial membranes. Double immunostaining using anti-Bax and anti-cytochrome c antibodies revealed that, in 100% of the cells with Bax in mitochondria, a diffuse cytosolic cytochrome c staining was observed, whereas cells with Bax in cytosol displayed a filamentous mitochondrial staining (1). Data presented in Fig. 1C confirm these published observations and show that, after Bax translocation, cytochrome c is released from mitochondria into the cytosol.

Mitochondrionally Localized Bax Forms Oligomers in the Membrane—Although the Bax molecule has mitochondrial targeting signals in its sequence, it remains unclear what factors keep it in cytosol in normal cells. The molecular modifications that Bax might undergo before or during translocation to mitochondria are also unknown. In vitro studies have shown that treatment of liposomes with Bax can permeabilize lipid membranes to allow transit of cytochrome c and dextrans (35). Calculation of the sizes of Bax-induced membrane pores has suggested that homo-oligomers of at least four Bax molecules are required to account for the results (35). These observations and other considerations suggested to us that translocated Bax in mitochondrial membranes might exist in the form of oligomers. To preserve the possible oligomeric state of Bax in the cytosol or mitochondrial membranes, we employed a battery of eight different bifunctional protein cross-linkers. Cross-linking was usually performed prior to solubilization of the membranes, since detergent treatment, by itself, can artificially induce dimerization of Bax with other Bax molecules and with Bcl-2 (26). These studies showed that Nonidet P-40 and Triton X-100, but not CHAPS, induced spurious homodimers and heterodimers (26). As a control to confirm these prior observations, and to test the efficacy of cross-linkers to stabilize putative oligomers, we also solubilized membranes in Nonidet P-40, Triton X-100, and CHAPS prior to cross-linking in some experiments.

In our initial studies, control and CCCP-treated cells were subjected to chemical cross-linking with a membrane-permeable linker (DSP) at different time points. Cytosol was collected and analyzed for Bax by SDS-PAGE and immunoblotting under non-reducing conditions. As shown in Fig. 2A, after prolonged incubation with CCCP, Bax disappeared from the cytosol. However, we did not detect any slow-moving Bax containing adducts in the cytosol at any of these time points. A photoactivatable cross-linker SANPAH, one of whose reactive groups can interact with any atom in the vicinity during cross-linking, also failed to demonstrate Bax adducts in the cytosol (data not shown), supporting the monomeric nature of cytosolic Bax. Analysis of DSP cross-linked membrane fractions of ATP-depleted cells showed that progressively greater amounts of Bax are present in membranes with increasing durations of ATP depletion (Fig. 2B). In contrast to cytosolic Bax (Fig. 2A), the membrane-inserted Bax formed slow-moving adducts (Fig. 2B, lanes 3–6). When the cross-linker was cleaved under reducing conditions with 50 mM DTT, only the 21-kDa species of Bax was present in these samples (Fig. 2C), suggesting that the slow-moving adducts indeed represent Bax-containing complexes. Interestingly, increasing the length of the cross-linkers from 6.4 to 16.1 Å permitted the demonstration of higher order oligomers possibly containing six or more molecules of Bax in membranes (Fig. 2D). The molecular sizes of these adducts obtained from various experiments were estimated to be multiples of ~21 kDa (Fig. 2E), suggesting the formation of Bax homo-oligomers. In order to rule out the possibility that pretreatment of cells with digitonin might induce Bax oligomer formation, membranes were prepared with or without digitonin treatment. As shown in Fig. 2F, digitonin is not responsible for Bax adduct formation in mitochondrial membranes of ATP-depleted cells.

Isoelectric Focusing Supports Homo-oligomerization of Bax in the Membrane—Although data presented in Fig. 2E suggest that Bax monomers make up membrane-bound oligomers, it does not rule out the possibility that Bax may form oligomers with other proteins of similar molecular size. To investigate this possibility, SDS-PAGE analysis was complemented with isoelectric focusing to distinguish homo-oligomers from hetero-oligomers. This approach assumes that Bax-interacting proteins must have isoelectric points different from Bax. The apoptotic Bax is an acidic protein with a theoretically estimated pI of 4.69, and this value correlated well with the measured value of ~4.75 for untreated Bax by isoelectric focusing (Fig. 3B). We employed two types of cross-linkers to analyze oligomeric Bax. The sulphydryl reactive agent DPDPB, which does not alter the net charge on reacting proteins, and the amine-reactive agent EGS, which lowers the pI of cross-linked proteins by reacting with basic (amine) groups, were
used. CHAPS extracted membrane oligomeric Bax treated without or with DPDPB (SDS-PAGE; Fig. 3A, lanes 1 and 2) focused at a pI of ~4.75 (Fig. 3B, lanes 1 and 2), a value similar to that of monomeric Bax from cytosol (Fig. 3B, lane 7). Since nonionic detergents but not CHAPS were shown to induce homo- and hetero-oligomerization of Bax (26), cytosolic Bax was exposed to either Triton X-100 or CHAPS prior to cross-linking with DPDPB. In either case, the cross-linked protein focused at the same pI as untreated monomeric cytosolic Bax (Fig. 3B, lanes 5-7). Results using the amine-reactive cross-linker EGS were similar, except that Bax migrated as a more acidic species at a pI of ~4.4 due to loss of free basic amine groups(s) in the protein. All other amine-reactive cross-linkers also reduced the pI of Bax (data not shown). Membrane-bound oligomeric Bax, stabilized by EGS and extracted with CHAPS (Fig. 3A, lane 3), and cytosolic Bax, artificially dimerized in the presence of Triton X-100 and stabilized by EGS (see Fig. 5A, lane 1), co-migrated during isoelectric focusing (Fig. 3B, lanes 3 and 4).

**Bcl-2 Expression Does Not Prevent Translocation of Bax to Mitochondria during ATP Depletion**—We have shown earlier by immunocytochemistry and Western blotting that Bcl-2 does not prevent Bax translocation to mitochondria following hypoxia or chemically induced ATP depletion in cultured proximal tubule cells, but is able to block the release of cytochrome c (1). Bax translocation is also a critical event in neuronal apoptosis and is not prevented by overexpression of Bcl-2 during nerve growth factor deprivation (36). However, Bcl-2 inhibited the release of cytochrome c, caspase activation, and cell death in these neurons (36). These results contradict other
published results where Bax migration into mitochondria was blocked by Bcl-2 overexpression (25, 37). The relative levels of total Bax in RPTC and Bcl-2 cells are not significantly different. With extended durations of ATP depletion, Bax translocation in Bcl-2 cells reaches similar levels as in RPTC (Fig. 4, lanes 1–5 and 6–10), as we have reported previously (1). The studies presented here had greater than 70% of total Bax translocated to membranes.

Bcl-2 Prevents Oligomerization of Translocated Bax in the Membrane without Forming Hetero-oligomers with Bax—We have reported before that translocated Bax molecules in membrane fractions of ATP-depleted Bcl-2 cells have little or no association with Bcl-2 as shown by cross-immunoprecipitation studies (1). When Bax antibodies were used, the immunoprecipitates did not contain Bcl-2; conversely, when Bcl-2 antibodies were used, only trace amounts of Bax were occasionally found in the precipitates. Even these trace amounts of Bax that were sometimes present in Bcl-2 immunoprecipitates are likely to represent low affinity nonspecific adsorption, or Bax/Bcl-2 heterodimers induced artificially by nonionic detergents (26, 38). We studied the issue further by using eight protein cross-linkers of different spacer lengths and chemical reactivities to represent low affinity nonspecific adsorption, or Bax/Bcl-2 associations when cross-linking was performed before detergent solubilization. Under these conditions as reported previously by Youle’s group (26), Bax/Bax, Bax/Bcl-2, and Bcl-2/Bcl-2 dimers are induced artificially by Nonidet P-40 and Triton X-100 but not by CHAPS. As the results in Fig. 5 show, Bax/Bax, Bax/Bcl-2, and Bcl-2/Bcl-2 adducts were readily demonstrable when cross-linking was done after Nonidet P-40 or Triton X-100 solubilization, but not BAX treatment. As the results in Fig. 5 show, Bax/Bax, Bax/Bcl-2, and Bcl-2/Bcl-2 adducts were readily demonstrable when cross-linking was done after Nonidet P-40 or Triton X-100 solubilization, but not CHAPS treatment. Finally, the spacer lengths of the five cross-linkers that we used vary between 6.4 and 19.9 Å, distances that should cover a wide range of separation of reactive groups in the partners. Together with the immunoprecipitation results, these observations provide important data that argue strongly for the validity of not only the positive observations with respect oligomeric Bax adducts, but also their prevention by Bcl-2, and the lack of demonstrable Bax/Bcl-2 associations when cross-linking was performed before detergent solubilization.

Detergent-induced Bax Homodimerization Is Inhibited by Bcl-2—It has been reported previously that detergents induce Bax homodimerization and Bax/Bcl-2 heterodimerization (26, 38). We have extended our studies to test whether Bax oligo-
gomizer in the presence of detergents. Whole cells with cytosolic Bax (normal RPTC) were extracted with the detergents Triton X-100, Nonidet P-40, and CHAPS and then subjected to chemical cross-linking with DSP. Bax analysis by immunoblotting showed that cytosolic Bax formed homodimers in the presence of detergents Triton X-100 and Nonidet P-40 (Fig. 5A). However, the zwitterionic detergent CHAPS failed to induce Bax homodimerization (Fig. 5A, lanes 2 and 5). These results agree completely with previous reports that showed differential effects of detergents on Bax dimerization (26, 38).

Since Bcl-2 prevented Bax oligomerization in the mitochondrial membrane, we tested whether Bcl-2 also interferes with detergent-induced Bax homodimerization. Normal Bcl-2 cells were solubilized with different detergents to allow membrane Bcl-2 and cytosolic Bax to interact with each other, cross-linked, and then analyzed by SDS-PAGE and Western blotting (Fig. 5B, lanes 1–6 (anti-Bax) and lane 7 (anti-Bcl-2)). In cell extracts from Bcl-2-overexpressing cells (Fig. 5B), Bax homodimerization was partially inhibited (Fig. 5, compare lanes 1 and 3 in panels A and B). Although Bcl-2 was able to form heterodimers with Bax in these detergents, the total amount of Bax/Bcl-2 heterodimers could not account for the entire decrease in Bax/Bax homodimer formation. Speculatively, it seems reasonable to consider that Bcl-2 may reduce Bax homodimerization by competing with Bax for space in detergent micelles. Although the results show that Bcl-2 has the ability to form heterodimers with Bax, they also show that the amounts of heterodimers that form are relatively sparse, considering the concentrations of the partners in the detergent, suggesting that they cannot form tight complexes. Further studies are clearly necessary to address the questions regarding Bax and Bcl-2 interactions among themselves and each other in detergents as well as in membranes. The Bax homodimers seen in Triton X-100 extracts without cross-linker (Fig. 5, lane 4 in panels A and B) are probably due to presence of oxidizing contaminants even in the membrane grade detergent. These dimers disappeared with pretreatment of extracts with 50 mM DTT for 30 min at 37 °C (but not boiling for 10 min). Similarly, the trace amounts of dimers seen after non-reducing SDS-PAGE of oligomerized membrane Bax not subjected to cross-linking (Figs. 2D and 3A, lane 1), which are probably due to incomplete dissociation and/or partial oxidation, also disappeared under reducing conditions (Fig. 2F, lanes 1 and 3).

In order to investigate the physical state of Bcl-2 in mitochondrial membranes, Bcl-2 cells were treated with DSP followed by membrane extraction with Triton X-100, Nonidet P-40, or CHAPS. Bcl-2 migrated as a monomeric protein on SDS-PAGE with (data not shown) or without cross-linking (Fig. 5C, lane 1). However, when the membrane extracts in above detergents were subjected to cross-linking, small amounts of Bcl-2/Bcl-2 homodimers were detected in Triton X-100 or Nonidet P-40 extracts (Fig. 5C, lanes 2 and 4) but not in CHAPS extract (Fig. 5C, lane 3). Together, these results show that detergents such as Nonidet P-40 and Triton X-100 can induce the formation of homo- and heterodimers of Bax and Bcl-2.

**Dimer-forming Detergents Reduce Higher Order Bax Oligomers to Dimers**—The data presented above indicate that the structural conformation of Bcl-2 and Bax in natural membranes is different from that in detergents. An important difference between detergents and membranes is that detergents predominantly form micellar structures, whereas membranes are organized as lipid bilayers with asymmetric distribution of proteins and lipids. We therefore tested whether detergent solubilization of membrane-inserted Bax would change its oligomeric properties. Stabilization of complexes by chemical cross-linking was carried out before or after detergent solubilization of membranes of ATP-depleted RPTC and Bcl-2-overexpressing cells. The data presented in Fig. 6A show that translocated Bax in natural membranes exists in oligomeric form. The higher orders of oligomers were reduced mainly to dimers when cross-linking was performed after solubilization with either Nonidet P-40 or Triton X-100 (Fig. 6A, compare lanes 1 and 2 or lanes 3 and 4). Lane 5 containing Triton X-100-solubilized membranes without cross-linker shows Bax dimers; as discussed earlier, dimer formation in this case is also attributable to incomplete dissociation and oxidants pres-
ent in Triton X-100 (Fig. 5). As shown before, Bax oligomerization was dramatically reduced in membranes from ATP-depleted Bcl-2-overexpressing cells (Fig. 6B, lanes 1 and 3). It is worth noting again that the Bcl-2 protein did not form either homo-oligomers or hetero-oligomers with Bax in such cells (Fig. 6B, lanes 1 and 3). However, if membranes had been solubilized with Nonidet P-40 and Triton X-100 before cross-linking, Bax/Bcl-2 heterodimers formed readily (Fig. 6B, lanes 2 and 4). The identity of Bax/Bcl-2 heterodimers was confirmed both by molecular weight calculation and Western blotting with anti-Bcl-2 antibodies (data not shown). In contrast, membranes solubilized in CHAPS before cross-linking still contained higher order Bax oligomers in RPTC (Fig. 6C, lane 1) but in ATP-depleted Bcl-2 cells, no Bcl-2 containing adducts were detected (Fig. 6C, lane 2). Controls with cross-linking followed by CHAPS extraction also showed similar results (Fig. 3A, lane 3). Overall, our results suggest that Bax oligomerization in the mitochondrial outer membrane is prevented under natural conditions (i.e. without prior exposure to detergents) by Bcl-2 without requirement for stable associations with Bax.

**VDAC or ANT Form Homodimers but Not Heterodimers with Bax**—Recently it has been reported that Bax may interact with an outer membrane protein, the voltage-dependent anion channel protein (also known as porin) to induce cytochrome c release (27). Another study reported that Bax might interact with an inner membrane protein, the adenine nucleotide translocator (28). This interaction has also been suggested to be an antecedent factor responsible for cytochrome c release. Therefore we searched for Bax and Bcl-2 interactions with VDAC or ANT in our ATP depletion model. Although our cross-linking and isoelectrofocusing studies have indicated no association of Bax with proteins other than itself, further studies were carried out to identify Bax-associated proteins. Membrane extracts were immunoprecipitated with antibodies to Bax or Bcl-2, and the resulting precipitates were analyzed for the presence of VDAC or ANT by immunoblotting. As shown in Fig. 7A, antibodies to Bax and Bcl-2 immunoprecipitated only Bax and Bcl-2, respectively, and did not bring down even traces of VDAC protein (Fig. 7A, lanes 2 and 3 and lanes 4 and 5). Our attempts to immunoprecipitate VDAC from nonionic detergent membrane extracts with four commercially available antibodies failed, though these antibodies could recognize VDAC in immunoblots (Fig. 7A, lane 1). However, when the membrane proteins were solubilized with SDS and renatured by diluting with Nonidet P-40-containing buffer, these same antibodies successfully immunoprecipitated VDAC (data not shown), suggesting that partial renaturation of VDAC exposes otherwise buried antibody-binding epitopes. Available antibodies against VDAC were therefore not usable for co-immunoprecipitation studies to identify association partners, at least in our model system. Conceivably, steric hindrance related to the complexity of the outer membrane protein microenvironment may also have been responsible for failed immunoprecipitation of VDAC with these antibodies. Immunoblotting of Bax and Bcl-2 immunoprecipitates for ANT also gave negative results (data not shown), suggesting that neither ANT nor VDAC is associated with translocated Bax in mitochondria. Immunoprecipitation under less stringent (150 mM NaCl washes only) as well as stringent (0.5 M NaCl washes included) conditions failed to reveal the presence of VDAC or ANT in the immunoprecipitates (data not shown). Therefore, chemical cross-linking approach was used to stabilize any weak interactions between proteins and identify Bax-associated proteins. Our results, presented in Fig. 7B, show that Bax and VDAC each can form
homo-oligomers; in the same fractions, Bax/VDAC associations were not observed as there were no adducts of intermediate size (54 kDa). Such associations between Bax and VDAC or ANT should have produced heterodimers in the size range of 52–56 kDa. We did not detect complexes in that size range in Bax containing mitochondrial membranes from ATP-depleted cells. Detection of VDAC homo-oligomers probably supports their role as channel-forming proteins. Additionally, Bcl-2/VDAC associations could not be demonstrated in Bcl-2-overexpressing control cells (Fig. 7B, lanes 8 and 9). Similarly, ANT protein also did not form associations with Bax or Bcl-2 (Fig. 7C). In control cells, DTBP did not cross-link ANT molecules, whereas DPDPPB, an agent with longer linker length, did (Fig. 7C, lanes 1 and 2 and lanes 5 and 6). On the other hand, in ATP-depleted cells, both DTBP and DPDPPB reacted with ANT and revealed dimers (Fig. 7C, lanes 3, 4, 7, and 8). This result suggests that ANT molecules, during ATP depletion, either come closer or undergo conformational change or both.

**FIG. 6.** Pre-exposure of Bax oligomers to nonionic detergents disintegrates higher order oligomers to dimers. Membranes from CCCP-treated (4 h) RPTC and Bcl-2 cells were either first subjected to cross-linking followed by extraction with nonionic detergents (CL+NP or CL+TX) or were solubilized first with different detergents and then subjected to cross-linking (NP+CL, TX+CL, or CH+CL). All samples (cross-linked with DSP) were analyzed under non-reducing conditions. A, higher order oligomers diminished markedly when membranes were exposed to nonionic detergents before being subjected to cross-linking (lanes 2 and 4) compared with cross-linking followed by extraction (lanes 1 and 3). Lane 5 represents Triton X-100 extract (membrane fraction) of CCCP-treated cells, without cross-linking, containing translocated Bax. Dimer represents artificial disulfide linkage not disrupted by SDS-PAGE under non-reducing conditions. B, in Bcl-2-overexpressing cell membranes, membrane-translocated Bax did not form higher order homo-oligomers or heterodimers with Bcl-2, as demonstrated by cross-linking followed by solubilization (lanes 1 and 3). Moreover, only trace amounts of Bax dimers could be seen, in contrast to the abundance of dimers and higher order oligomers that form in membranes in wild type (RPTC) cells (A). However, when membranes were exposed to the nonionic detergents Nonidet P-40 (lane 2) or Triton X-100 (lane 4) before cross-linking, Bax/Bcl-2 heterodimers appeared. The identity of the faint bands seen spanning below the 31-kDa region is not known. C, CHAPS solubilization prior to cross-linking still preserved Bax oligomers, which formed after translocation to the membrane (lane 1). Results with cross-linking followed by CHAPS solubilization are identical (see Fig. 3A, lane 3). In Bcl-2, cell membranes containing translocated Bax, Bcl-2 did not form any slow moving adducts (lane 2). CH, CHAPS; NP, Nonidet P-40; TX, Triton X-100.
to allow cross-linking by DTBP. Our failure to identify intermediate forms of ANT/Bax or ANT/Bcl-2 complexes with four other cross-linkers of different spacer arm lengths and reactive specificities, including the nonspecific highly reactive photo cross-linker SANPAH (data not shown), together with unequivocal negative immunoprecipitation results, argues against the necessity for such interactions for the function of Bax or Bcl-2 proteins.

**Isoelectric Focusing Analysis to Identify Homo-oligomerization Versus Hetero-oligomerization of Bax**—The calculated values of isoelectric points (pI) of rat Bax (4.69) and human Bcl-2 (7.32) are close to experimental values of 4.76 (rat Bax) and 7.39 (human Bcl-2, see Fig. 8A). The calculated pI values of the proteins that have been reported to interact with Bax, e.g., VDAC (9.04), ANT (10.54), Bcl-2 (7.32) and proteins that are known to be released by Bax from the mitochondria, e.g., cytochrome c (10.39), adenylate kinase (9.86), and apoptosis-inducing factor (9.63) suggest that all of these proteins are basic in nature. In contrast, Bax is an acidic protein (pI 4.75) both in RPTC (see Fig. 3) and Bcl-2-overexpressing cells (Fig. 8A, lanes 1–3) before or after translocation to membrane. Bcl-2, in normal cells, has two fast moving, minor isoforms with pI of 6.61 (71x342).
Bax Oligomerization

**DISCUSSION**

Regulation of apoptosis by the Bcl-2 family of proteins occurs primarily at the mitochondrial outer membrane and involves mitochondrial permeabilization or its prevention. The studies presented in this paper are attempts to understand the mechanisms by which Bax induces cytochrome c efflux from mitochondria of ATP-depleted cells. Although the signaling mechanisms responsible for Bax translocation during ATP depletion remain unclear, the experiments reported here have revealed important insights into the physical states and associations of Bax molecules after they have been inserted into mitochondrial membranes. Using chemical cross-linkers, we were able to detect higher order homo-oligomers of Bax in membrane fractions of ATP-depleted cells. The presence of Bax oligomers from tetramers to decamers (Figs. 2, 3, 4, and 6) in the membrane fraction suggests that Bax may be able to form large structures with potential to allow the passage of proteins at least of the size of cytochrome c (12 kDa). Recently, it has been demonstrated that at least 4 molecules of Bax can form a pore of size 22 Å that is capable of transporting cytochrome c, a molecule with a Stokes diameter of 17 Å (35). Similar oligomers were identified with recombinant Bax protein in the presence of octyl glucoside (42). Isoelectric focusing analysis of these cross-linked oligomers suggests that Bax multimers contain homogeneous populations of Bax molecules (Figs. 3 and 8). Isoelectric focusing methodology was described under “Experimental Procedures.” A, CHAPS extract of membranes from ATP-depleted and control Bcl-2, cells were subjected to IEF with or without cross-linking, as described under “Experimental Procedures” (−CL, no cross-linker; CL, 1, DPDPB; CL 2, EGS). The sulfhydryl-reacting cross-linker, DPDPB, does not change the pl of Bax or Bcl-2 (lanes 2 and 5), whereas the amine-reactive cross-linker, EGS, changes the pl of these proteins (lanes 3 and 6). Bcl-2 has two minor isoforms with pl lower than the predominant form indicated by arrowheads (lane 4). After DPDPB cross-linking, these two isoforms were not seen, suggesting possible cross-linking of these forms to non-soluble cell structures with free-sulfhydryl groups. In either case, complexes with pl greater or lesser than the pl of Bax or Bcl-2 were not detected with or without cross-linking, suggesting lack of interaction with other proteins including VDAC and ANT. B, control Bcl-2, cells were solubilized in Nonidet P-40 followed by cross-linking with DPDPB. Detergent-induced Bax/Bcl-2 heterodimers (see inset for sample analyzed by SDS-PAGE and immunoblotting) were detected by immunoblotting after IEF. Both α-Bax and α-Bcl-2 antibodies were utilized sequentially to identify Bax and Bcl-2 in the immunoblot. The pl of the Bax/Bcl-2 complex is the average of Bax and Bcl-2 pl values. CL 1, DPDPB; CL 2, EGS; −CL, no cross-linker.
cytosolic Bax with itself or Bcl-2, as demonstrated by cross-linking, suggesting that detergents were altering the conformation of cytosolic Bax (Fig. 5). However, CHAPS, a zwitterionic detergent, did not induce Bax or Bcl-2 oligomerization, which suggests that this detergent has actions different from those of nonionic detergents with respect to its ability to modify Bax or Bcl-2 molecules. These results confirm earlier work from Youle’s group (26, 38) and suggest that Bax monomers move to mitochondrial membranes in response to unknown stimuli and form homo-oligomers in situ. Our results also suggest that, although there may be similarities between lipid bilayers and detergent micelles in their effects on Bax with respect to their shared ability to induce and maintain oligomers, there are also key differences. When cross-linking of membrane proteins was carried out after membrane solubilization in Nonidet P-40 or Triton X-100, multimers of Bax were either absent or present in trace amounts, the vast majority of Bax being present as dimers and monomers (Fig. 6A). This suggests that only planar membrane bilayers allow Bax oligomerization to higher order structures, and that nonionic detergents might have dissociated these pre-existing Bax multimers. In contrast, CHAPS, which does not induce oligomerization of cytosolic Bax, also failed to dissociate higher order Bax oligomers in mitochondrial membranes (Fig. 6C).

In contrast to results obtained by others, we have shown that Bcl-2 overexpression does not abolish Bax translocation to mitochondria (Fig. 4A). The results presented in this paper demonstrate that Bcl-2 cannot block Bax association with mitochondria in the ATP depletion model. However, Bcl-2 can completely prevent Bax oligomerization in the mitochondrial outer membrane (Figs. 4 (B and C) and 6B). Thus, at least in our experimental model, Bcl-2 may inhibit cytochrome c release and protect cells by preventing oligomerization of Bax rather than by blocking Bax insertion into mitochondrial membrane. Moreover, we also found that Bax translocation to mitochondria in rat proximal tubule cells is independent of apoptotic stimuli. UV (80 J/m²) exposure also induces Bax translocation in both RPTC and Bcl-2 cells but oligomerization is seen only in RPTC but not in Bcl-2 cells. This suggests that prevention of Bax translocation is not a necessary prerequisite for the protective actions of Bcl-2 in mitochondrial membranes.

Our results suggest that prevention of Bax homo-oligomerization by Bcl-2 does not involve direct interactions of Bcl-2 with Bax, at least to an extent of close proximity and affinity.
that could have been revealed by co-immunoprecipitation, or demonstrable by eight different cross-linkers with varying spacer arms (6.4–19.9 Å) and reactivities (imido and NHS esters for amines, pyridyldithio for sulfhydryls, and photolabile nitrophenylazide for non-selective linking to atoms in the vicinity). All of these cross-linkers were able to form Bcl-2 adducts, visualized as Bcl-2 homodimers and Bcl-2/Bax heterodimers, only when pretreated with nonionic detergents prior to cross-linking; but failed to detect such adducts when membranes were cross-linked prior to detergent extraction. Together with immunoprecipitation studies, our results suggest strongly that mechanisms other than direct Bcl-2/Bax interactions must be involved in Bcl-2 protection against Bax cytotoxicity. How then, could Bcl-2 prevent Bax-induced leakage strongly that mechanisms other than direct Bcl-2/Bax interaction with translocated Bax from Bcl-2-overexpressing cells (Fig. 9). Our failure to observe Bax oligomers in membranes with translocated Bax from Bcl-2-overexpressing cells suggests that partially inserted Bax may not have undergone conformational changes required to form oligomers/channels, perhaps by steric constraints imposed by abundant Bcl-2 molecules occupying the same microenvironment(s). This explanation demands that Bax and Bcl-2 share some critical lipid domains, and possibly cooperating proteins in mitochondrial membranes. Conceivably, these membrane domains and cooperating proteins are required for full Bax insertion and function, made possible by exposure of previously “masked” sequences. If the “shared” domains are saturated with Bcl-2, Bax molecules are either denied access totally, or provided access for limited insertion by steric restraints that prevent assumption of configurations necessary for oligomer/channel formation. This hypothesis is consistent not only with our present findings, which show Bax insertion without oligomerization, but also with the findings of other laboratories, which have reported reduction and even prevention of Bax translocation by Bcl-2 (37, 43).

Previous reports have suggested that Bax heterodimerization with mitochondrial outer membrane porin (VDAC) or inner membrane protein ANT may be involved in the release of cytochrome c or other inter-membrane space proteins. However, we failed to detect associations of Bax with either ANT or VDAC by immunoprecipitation of extracts from cells with mitochondrially translocated Bax. High salt washes during immunoprecipitation were deliberately avoided to encourage low affinity or nonspecific protein interactions in some experiments; nevertheless, we failed to co-immunoprecipitate ANT or VDAC with Bax. Moreover, eight different cross-linkers of diverse spacer lengths and chemical reactivities also failed to reveal adducts between Bax and VDAC or ANT. On the other hand, these agents readily demonstrated homo-oligomers of ANT and VDAC. Additionally, the pl of monomeric Bax in the cytosol and oligomeric translocated Bax in membranes, with or without stabilization by cross-linkers, were identical. This supports the idea that Bax oligomers were homogeneous in composition and contained Bax exclusively. It is particularly instructive to compare overall the behavior of oligomeric Bax, membrane-bound Bax extracted with CHAPS (which does not induce oligomerization by itself), monomeric Bax in the cytosol exposed to CHAPS with or without cross-linker, cross-linked cytosolic Bax after dimerization by Triton X-100, and untreated natural Bax. The results show that neither the detergent nor the cross-linker DPDPB had altered the net charge of the protein species. Under these conditions, the identity of pl among all the species argues forcefully for the formation of Bax homo-oligomers in membrane fractions of ATP-depleted cells with translocated protein.

In summary, our results are consistent with a model (Fig. 9) of Bax-dependent mitochondrial permeabilization in which formation of Bax oligomers in the outer mitochondrial membrane triggers the release of cytochrome c. The anti-apoptotic protein, Bcl-2, when overexpressed, may either interfere with Bax insertion into membranes (37, 43), or prevent Bax oligomerization in the membrane following ATP depletion or UV irradiation by mechanisms related to steric exclusion from critical microdomains in mitochondrial membranes. This model also considers the possibility of oligomeric Bax somehow inducing pore formation by other outer membrane proteins to permeabilize cytochrome c. In the light of recent data, the latter possibility is considered unlikely, suggesting that Bax oligomers by themselves in liposomes are capable of transporting cytochrome c across the lipid bilayer (35). According to our model, Bcl-2 prevents such oligomerization by physically interfering with complete insertion of Bax into the membrane, a requirement for oligomerization, without forming stable heteromeric complexes. Therefore, partially inserted but loosely bound Bax, in Bcl-2-loaded mitochondria, may quickly equilibrate with cytosolic Bax in other models of apoptosis where Bax association with mitochondria in Bcl-2-overexpressing cells is seemingly reduced. Future studies on purified, stably cross-linked multimers of Bax and systems that permit regulated expression of Bcl-2 should help to clarify these issues.

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