Association of Bax and Bak Homo-oligomers in Mitochondria

Valery Mikhailov, Margarita Mikhailova, Kurt Degenhardt‡, Manjeri A. Venkatachalam, Eileen White†§, and Pothana Saikumar¶

From the Department of Pathology, The University of Texas Health Science Center, San Antonio, Texas 78229 and the §Center for Advanced Biotechnology and Medicine, Howard Hughes Medical Institute, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey 08854

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Bax REQUIREMENT FOR Bak REORGANIZATION AND CYTOCHROME c RELEASE*

ATP depletion induced by hypoxia or mitochondrial inhibitors results in Bax translocation from cytosol to mitochondria and release of cytochrome c from mitochondria into cytosol in cultured rat proximal tubule cells. Translocated Bax undergoes further conformational changes to oligomerize into high molecular weight complexes (Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A., and Saikumar, P. (2001) J. Biol. Chem. 276, 18361–18374). Here we report that following Bax translocation in ATP-depleted rat proximal tubule cells, Bak, a proapoptotic molecule that normally resides in mitochondria, also reorganizes to form homo-oligomers. Oligomerization of both Bax and Bak occurred independently of Bid cleavage and/or translocation. Western blots of chemically cross-linked membrane extracts showed nonoverlapping “ladders” of Bax and Bak complexes in multiples of ~21 and ~23 kDa, respectively, consistent with molecular homogeneity within each ladder. This indicated that Bax and Bak complexes were homo-oligomeric. Nevertheless, each oligomer could be co-immunoprecipitated with the other, suggesting a degree of affinity between Bax and Bak that permitted co-precipitation but not cross-linking. Furthermore, dissociation of cross-linked complexes by SDS and reconstitution prior to immunoprecipitation did not prevent reassociation of the two oligomeric species. Notably, expression of Bcl-2 prevented not only the oligomerization of Bax and Bak, but also the association between these two proteins in energy-deprived cells. Using Bax-deficient HCT116 and BMK cells, we show that there is stringent Bax requirement for Bak homo-oligomerization and for cytochrome c release during energy deprivation. Using Bax-deficient BMK cells we further show that Bak deficiency is associated with delayed kinetics of Bax translocation but does not affect either the oligomerization of translocated Bax or the leakage of cytochrome c. These results suggest a degree of functional cooperation between Bax and Bak in this form of cell injury, but also demonstrate an absolute requirement of Bax for mitochondrial permeabilization.

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‡ To whom correspondence should be addressed: Dept. of Pathology, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229. Tel.: 210-567-6597; Fax: 210-567-2367; E-mail: saikumar@uthscsa.edu.

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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 (24). This was attributed to pore formation by Bax homo-oligomerization (16). Bax oligomerization as well as mitochondrial outer membrane permeabilization was prevented by Bcl-2 without forming physical complexes with Bax (16). Here we report that Bak, another proapoptotic member of the Bcl-2 family, exists as part of a large protein complex of unknown composition in mitochondria of normal cells, but undergoes rearrangement to form homo-oligomeric complexes following Bax insertion into mitochondria of hypoxic/ATP-depleted cells. Our data suggest functional dependence between Bax and Bak and strong association between Bax and Bak complexes in mitochondria. In addition they also demonstrate a stringent Bax dependence to affect mitochondrial permeabilization. Accordingly, selective Bak deficiency delayed the kinetics of Bax translocation, but did not prevent Bax oligomerization or cytochrome c release. On the other hand, selective Bax deficiency prevented both Bak oligomerization and cytochrome c release.

**EXPERIMENTAL PROCEDURES**

**Materials**—DMEM, 1 Ham’s F-12/DMEM, McCoy’s 5A medium, and minimum essential medium were from Invitrogen. Antibodies were from clone 2G8.B6 kindly provided by Dr. R. Jemmerson of University of Massachusetts, Boston, MA. DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; t-Bid, truncated Bid.

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RESULTS

Bax Translocation, and Reorganization/Oligomerization of Bax and Bak during ATP Depletion—ATP depletion by hypoxia or treatment with CCCP, a mitochondrial uncoupler, was shown to cause Bax translocation to mitochondria and release of cytochrome c into cytosol in cultured rat kidney proximal tubule cells (16, 24). The BMK cells deficient in both Bax and Bak showed resistance to tumor necrosis factor-α-induced cell death (25). However, cells deficient in Bax or Bak alone were susceptible to tumor necrosis factor-α-induced cell death suggesting that Bak can substitute for Bax in forming mitochondrial pores to release cytochrome c. This prompted us to further investigate the role of Bax and Bak in outer membrane permeability of mitochondria in energy-deficient cells. Double immunolabeling of RPTC with anti-Bak (green) and anti-Bax (red) revealed that Bak is constitutively present in mitochondria whereas Bax is predominantly localized in the cytosol of normal cells (Fig. 1A, panel 1). ATP depletion by CCCP resulted in co-localization of both Bak and Bax in the mitochondria (Fig. 1A, panel 2). We have shown earlier that following Bax translocation, cytochrome c is released into cytosol (16, 24). To find out whether the development of permeability transitions can explain cytochrome c release after prolonged exposure to CCCP, cells depleted of ATP by CCCP were allowed to recover in complete growth medium without CCCP, but in the presence of z-VAD, a caspase inhibitor to block downstream events of apoptosis (Recovery). Both groups of cells as well as control cells (Control) were loaded with rhodamine 123 for 10–15 min and photographed using fluorescence microscopy. C, following incubation of RPTC with 15 μM CCCP for 0, 1, 2, 3, or 4 h, cells were treated with the cleavable membrane-permeable cross-linker dithiobis(succinimidyl propionate) (1 min). Membrane fractions were obtained as described under “Experimental Procedures,” and analyzed for Bak (lanes 1–5) or Bak (lanes 6–10) by Western blotting under nonreducing conditions. Prolonged exposure to CCCP resulted in progressive accumulation of Bak in the membrane fraction as slow moving complexes (lanes 3–5). Similarly, slow moving Bak complexes appeared after ATP depletion in parallel with Bak accumulation in mitochondria (lanes 6–10). Closed arrowheads indicate Bak oligomers and open arrowheads indicate Bak oligomers. D, membrane extracts of ATP-depleted cells (4 h CCCP) after incubation with the noncleavable cross-linker EGS were analyzed by Western blotting under reducing conditions. The molecular weights of Bak and Bak complexes were calculated by plotting their migrations against migration of molecular weight standards in semilogarithmic plots from several experiments. Two representative lanes of Bak ladders and Bak ladders are shown and they show homo-oligomers of Bak and Bax, respectively. E, time course analysis of Bid distribution by Western blotting in the cytosol and membranes of ATP-depleted cells.
both control and recovering cells were able to a mount potential showing of perinuclear mitochondrial distribution of rhodamine fluorescence (Fig. 1B, panels 1 and 3). On the other hand, in CCCP-treated cells, rhodamine 123 remained in the cytosol indicating loss of mitochondrial potential (Fig. 1B, panel 2). Experiments done in parallel showed that cells treated similarly with CCCP had leaked cytochrome c into cytosol without or with an additional period of recovery (not shown). The ability of mitochondria to accumulate rhodamine 123 despite having leaked cytochrome c precluded the possibility that permeability transitions had occurred, because the development of transitions is inconsistent with the ability of membranes to maintain a barrier to the free diffusion of protons.

To identify Bax-Bak interactions, cells were fractionated into cytosolic and membrane fractions (16) followed by chemical cross-linking with dithiobis(succinimidyl propionate) or EGS. Western blotting of cross-linked proteins showed that progressively greater amounts of Bax translocated to mitochondria during increasing durations of ATP depletion and oligomerized into dimers and higher oligomers in multiples of ~21 kDa, the monomer (16) (Fig. 1C, lanes 1–5). Concurrently, mitochondrial Bak, normally present as monomers as well as very large protein complexes, rearranged to form Bak oligomeric “ladders” (Fig. 1, C and D). As in the case of Bax (16), the calculated molecular weights of Bak complexes in these ladders correspond to multiples of ~23 kDa, the monomer (Fig. 1D, lane 4). This suggests that these newly formed complexes of Bak are homo-oligomers. The mobility and molecular sizes of Bak oligomers were quite distinct from those of Bax oligomers (Fig. 1, C and D). The large Bak complexes greater than 250 kDa that were observed in normal cells at the top of the gel (Fig. 1D, lane 3) were diminished in amount during ATP depletion with concomitant appearance of Bak oligomers (Fig. 1D, lane 4). It has been reported that Bak oligomerization is associated with t-Bid translocation to mitochondria after Bid cleavage by caspase-8 (17). Analysis of Bid protein in RPTC during ATP depletion (Fig. 1E) indicated no significant change of Bid protein levels in the cytosol. The small amount of Bid detected in membrane fractions of control cells was unchanged during ATP depletion. Furthermore, we did not detect cleaved products of Bid such as t-Bid (~15 kDa) in the membrane fraction, suggesting that Bid is not involved in Bax translocation and oligomerization.

Bax translocation was observed also in HeLa cancer cells that had been subjected to hypoxia or ATP depletion. The data presented in Fig. 2A show Bax translocation and release of cytochrome c from mitochondria into cytosol during hypoxia in HeLa cells. As in the case of RPTC, hypoxia did not lead to Bid cleavage (Fig. 2A) or affect its localization. Chemical cross-linking of membrane proteins in HeLa cells showed oligomerization of translocated Bax (Fig. 2B, lane 4′) and dimerization of Bak (Fig. 2B, lane 4). Bak reorganization was mainly seen as Bak dimer in HeLa cells; this appears to be because of overshadowing of Bak oligomers by very large amounts of Bak containing protein complexes of heterogeneous molecular size in control as well as in hypoxic cells (Fig. 2B, lanes 2 and 4). Formation of Oligomeric Complexes of Bax and Bak—We used size exclusion chromatography to characterize Bax and Bak protein complexes in RPTC. Total cell lysates in 2% CHAPS lysis buffer from normal and ATP-depleted RPTC were fractionated on a Superose 6 gel filtration column and eluted fractions were analyzed by Western blotting. Results presented in Fig. 3A indicated that Bax is monomeric in normal cells with a peak elution at about 25 kDa. In contrast, Bax from ATP-depleted cells eluted in fractions with apparent molecular weights between 25,000 and 134,000 with a peak at about 440,000 consistent with the formation of large Bax complexes (Fig. 3A). On the other hand, Bak eluted in a broad range between 43 to 5000 kDa even in normal cells, with a peak at about 232 kDa (Fig. 3B). The distribution of Bak as part of a large complex in mitochondria of normal cells was evident also after protein cross-linking; Bak adducts of >250 kDa size were observed (Fig. 1C). At present, the identity of protein(s) associated with Bak in mitochondria is unknown. ATP depletion induced a small but significant alteration in Bak elution profile with a peak at about 440 kDa (Fig. 3B). The overlapping elution profiles of Bax and Bak suggest possible association between these proteins in the mitochondria of ATP-depleted cells.

Immunoelectron microscopy confirmed the translocation and localization of Bax in mitochondrial membranes in the form of complexes. Bax was seen exclusively in the cytosol of normal cells but clustered around mitochondria in apoptotic cells after ATP depletion and repletion induced by reincubation in growth.
Oligomerization of Bax and Bak Is Inhibited by Bcl-2—We have shown earlier that Bcl-2 overexpression prevents Bax oligomerization and cytochrome c release in RPTC after hypoxia or ATP depletion (16). We have now tested the effect of Bcl-2 on Bak oligomerization using chemical cross-linking and gel filtration. As shown in Fig. 4A, Bcl-2 overexpression prevented the rearrangement of both translocated Bax and mitochondrial Bak during ATP depletion induced by CCCP. By gel filtration, alterations in the elution profile of Bax in ATP-depleted Bcl-2 cells were only modest (Fig. 4B). On the other hand, the Bak elution profile was compressed following CCCP treatment of Bcl-2 cells (Fig. 4C) as in the wild type RPTC (Fig. 3B). The compression of elution profiles corresponded to reduction in the amounts of normally present large Bak adducts retained at the top of the gel after chemical cross-linking (Fig. 4A, lanes 6 and 8). Nevertheless, smaller Bak oligomers did not form in ATP-depleted Bcl-2 overexpressing cells relative to controls (Fig. 4A, lane 8). These results suggest that Bcl-2 inhibits not only the oligomerization of Bax following translocation of the protein, but also the rearrangement of mitochondrial Bak to form small homo-oligomers.

**Bax Oligomers Are Associated with Bak Oligomers—** Whereas gel filtration studies have helped to identify the formation of large protein complexes, they failed to characterize the organization of these complexes. Therefore, chemical cross-linking along with immunoprecipitation was undertaken to clarify how Bax and Bak complexes are organized during ATP depletion. For immunoprecipitation, CHAPS extracts were used because nonionic detergents such as Nonidet P-40 and Triton X-100 induce conformational change and oligomerization of Bak (16, 29). The anti-Bax antibody (1D1) recognizes a buried epitope of Bax in normal cells and precipitates little or no Bax (Fig. 5A, lane 7 and 9, bottom panel) (24). However, Bax is precipitated by the anti-Bax antibody in ATP-depleted RPTC and Bcl-2 cells (Fig. 5A, bottom panel, compare lanes 8 and 10 with lanes 7 and 9) because of Bak conformational changes that result in membrane translocation. A modest but significant increase of Bak precipitation was seen with anti-Bak antibodies as described under “Experimental Procedures.” A, the scanned elution profile of Bak in normal (RPTC/Con) and ATP-depleted cells (RPTC/CCCP). B, the scanned elution profile of Bak from normal (RPTC/Con) and ATP-depleted cells (RPTC/CCCP). C, EM immunochemistry of Bax in normal and apoptotic RPTC. ATP depletion followed by recovery in complete growth medium induced apoptosis because of resynthesis of glycolytic ATP. Arrowheads represent cytosolic Bax in panel 1. M represents mitochondria in panel 2.
immunoprecipitations from ATP-depleted cells contained Bax (Fig. 5A, bottom panel, lane 2). Therefore, co-immunoprecipitation results indicated possible association between Bax and Bcl-2 in the mitochondria of ATP-depleted wild type RPTC (Fig. 5A, lanes 2 and 8). As expected, overexpression of Bcl-2 inhibited co-immunoprecipitation of Bax and Bak from ATP-depleted cells (Fig. 5A, lanes 4 and 10). Furthermore, Bcl-2 was not co-precipitated with either Bax antibodies or Bak antibodies (not shown) suggesting lack of physically stable association between pro-apoptotic Bcl-2 or Bax with anti-apoptotic Bcl-2. To detect Bax-Bak oligomers with increased sensitivity, chemically cross-linked extracts were also subjected to co-immunoprecipitation. Interestingly, distinct ladders of Bax dimers, trimers, and higher order oligomers were co-precipitated with anti-Bak (Fig. 5B, lane 6), and Bak dimers, trimers, and tetramers were co-precipitated with anti-Bak in ATP-depleted RPTC (Fig. 5B, lane 2) confirming the association between Bax and Bak oligomers.

**Dissociation and Reassociation of Bax and Bak Oligomers**—The presence of nonoverlapping ladders of Bax and Bak raised the question of how Bax homo-oligomers would interact with Bak homo-oligomers. To address this question, in some experiments, mitochondrial membranes from ATP-depleted cells were extracted with SDS buffer (0.35%) with or without prior chemical cross-linking (labeled +SDS in Fig. 6). The SDS extracts were heated to 70 °C for 10 min to dissociate noncovalently interacting molecules. Proteins were renatured by 10-fold dilution of SDS in the presence of 2% CHAPS at room temperature. After SDS dilution, proteins were immunoprecipitated with either anti-Bak or anti-Bax antibodies. In another group of experiments, immunoprecipitation was performed without prior SDS treatment (labeled −SDS in Fig. 6). Western blotting analysis of Bak (or Bax) immunoprecipitates of noncross-linked mitochondrial membranes revealed that little or no Bax (or Bak) is co-precipitated if they had been exposed to SDS prior to treatment with the precipitating antibody (shown for Bak immunoprecipitate in Fig. 6A). Thus, in the absence of chemical cross-linking, all complexes of Bax and Bak were separated into monomers during SDS treatment. Failure to co-precipitate Bax with Bak from dissociated complexes suggests that Bax and Bax monomers did not reassociate with each other after exposure to SDS. On the other hand, immunoprecipitation of Bak from chemically cross-linked mitochondrial proteins after SDS denaturation and dilution in CHAPS yielded Bax oligomers containing three or more molecules (Fig. 6B, lanes 5 and 6). These immunoprecipitates contained little or no Bax monomers and dimers (Fig. 6B, lane 6) relative to conditions where they had not been previously exposed to denaturation with SDS (Fig. 6B, lane 5). Of interest, there were modest amounts of Bax-Bak heterodimers in the precipitates that resisted dissociation by SDS. However, higher order Bax-Bak oligomers could not be identified. Anti-Bax antibodies precipitated all forms of Bax including monomers and dimers, with or without SDS denaturation and dilution in CHAPS (Fig. 6B, lanes 3 and 4). In the absence of interaction between monomeric forms of Bax and Bak, this result suggests that all forms of Bak interact with oligomeric Bax.

The most important inference from these observations on the effect of SDS exposure prior to immunoprecipitation of cross-linked Bax and Bak is that regardless of prior SDS treatment, co-precipitated ladders of Bax and Bax are nonoverlapping with respect to the molecular sizes of the respective oligomers (Fig. 6B, lanes 1, 2 and 3, 4). The results pose a paradox with respect to how Bax and Bak oligomers are formed and how they associate with each other. It seems apparent that the observations can only be explained by the formation of largely homogeneous Bax and Bax homo-oligomers in ATP-depleted mitochondrial membranes. The propensity of these oligomers to co-precipitate may indicate a degree of affinity between Bax and Bax oligomers that cannot be preserved by cross-linking, but one which may be compatible with a role for Bax translocation and homo-oligomerization in subsequently inducing the recruitment and formation of Bak oligomers. Thus, oligomerization of translocated Bax may be a pre-requisite to interact with Bak and cause Bak reorganization.

**Functional Interdependence of Bax and Bak**—To investigate the role of Bax in Bak reorganization and cytochrome c release, we have subjected Bax(+/-) and Bax(-/-) colon cancer cells to...
ATP depletion by CCCP. Bax was totally absent in Bax(−/−) cells. Unlike RPTC or HeLa cells, Bax(+/−) cells contained 30–40% of total Bax in mitochondria, and the remainder was present in the cytosol of normal cells (Fig. 7A, lanes 1 and 2). When mitochondria isolated from Bax(+/−) cells were incubated for 30 min at 30°C, the majority of mitochondrially associated Bax was released into the medium (Fig. 7A, lane 4). This observation indicates a loose association of Bax with mitochondria in normal Bax(+/−) cells. Moreover, mitochondrially associated Bax in normal cells did not occur in oligomeric form as shown by chemical cross-linking (Fig. 7B, lane 10). Upon incubation of Bax(+/−) cells with CCCP, formation of Bax oligomers was evident following chemical cross-linking, with concomitant release of cytochrome c (Fig. 7, B, lanes 11 and 12, C, lanes 5 and 6). Bak was abundant in both Bax(+/−) and Bax(−/−) cells (Fig. 7B, lanes 1–6). As in the case of HeLa cells (Fig. 2B), CCCP-induced Bak oligomers were seen largely in the form of dimers after cross-linking in Bax(+/−) cells (Fig. 7B, lanes 5 and 6). With ATP depletion, Bak dimers increased significantly in Bax(+/−) cells, but not in Bax(−/−) cells (Fig. 7B, lanes 1–6). These results are most consistent with a role for Bax in the reorganization/oligomerization of Bak that takes place during ATP depletion. Regardless of these considerations, the results also showed that there is an absolute requirement for Bax expression to release cytochrome c in significant amounts during ATP depletion (Fig. 7C, compare lanes 2, 3 and lanes 5, 6). The small amount of cytochrome c release seen in Bax(−/−) cells can be attributed to the fragility of energy-deprived cells subjected to plasma membrane permeabilization methods. A potential role of permeability transitions in the release of cytochrome c during ATP depletion by CCCP or hypoxia was ruled out by the ability of affected mitochondria to mount potential and accumulate potentiometric dyes after removal of CCCP (Fig. 1C) or reoxygenation (24) in complete growth medium. Moreover, our results showing little or no release of cytochrome c in Bax(−/−) cells after prolonged treatment with CCCP (Fig. 7C) also rule out a role for the permeability transition in this phenomenon.

To determine whether Bax can independently oligomerize and release cytochrome c in the absence of Bak, we used transformed baby mouse kidney cells (25) derived from wild type (Bax(+/−)/Bak(+/+) Bax(Bak−/−), Bak (Bak−/−)/Bak(Bak−/−)) mice. We observed progressive translocation of Bax to mitochondria with increasing durations of ATP depletion in wild type BMK cells (Fig. 8A, lanes 1–5). However, Bax(−/−) cells showed delayed kinetics of Bax translocation (Fig. 8A, lanes 6–10). After 3 h of ATP depletion in wild type cells, the vast majority of cytosolic Bax had translocated to mitochondria and >90% of cells had released cytochrome c into the cytosol (Fig. 8B, panel 1). On the other hand, in Bak knockout cells, Bax translocation was significantly delayed (Fig. 8A, lanes 6–10). Corresponding to this, less than 20% of cells showed cytochrome c release (Fig. 8B, panel 3). In contrast, both Bax knockout and Bak/Bak double knockout cells showed resistance to cytochrome c release (Fig. 8B, panels 2 and 4). Chemical cross-linking of membranes revealed that translocated Bax was oligomerized into dimers and higher order oligomers in energy-deprived wild type BMK cells (Fig. 8C, lane 2). Similarly treated Bak(−/−) cells also showed Bax oligomerization (Fig. 8C, lane 6). These results clearly suggest that Bax does not require Bak to oligomerize after translocation to mitochondria. However, Bax deficiency seems to affect the kinetics of Bax translocation probably because of molecular changes in mitochondria that could have occurred because of Bak deficiency. Similarly, mitochondrial Bak, normally present as monomers as well as larger protein complexes bigger than dimers, rearranged to form Bak homo-oligomeric ladders in wild type BMK cells during energy deprivation (Fig. 8D, compare lanes 1 and 2). As in the case of Bax, the calculated molecular weights of rearranged Bak complexes in these ladders correspond to dimers, trimers, and higher order homo-oligomers. In contrast, Bax knockout cells failed to show Bak rearrangement to form homo-oligomers even after prolonged incubation under ATP-depleted conditions (Fig. 8D, lanes 5 and 6). These results clearly sug-
FIG. 7. Requirement of Bax for Bak oligomerization and cytochrome c release in Bax<sup>−/−</sup>, Bax<sup>+/−</sup> colon cancer cells. A, Western blot analysis of cytosol (Cyto, lane 1) and mitochondria (Mito, lane 2) obtained by differential centrifugation of Bax<sup>+/−</sup> cell homogenates (16), for Bax and Bak. Proteins were loaded in proportional amounts (Cyto:Mito, 3:1) on SDS-PAGE. Isolated mitochondria from normal Bax<sup>−/−</sup> cells were incubated in isonicotinic buffer (10 mM HEPES, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 125 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM succinate, and 1 mM ADP, pH 7.4) at 0 °C (lane 3) or 30 °C (lane 4) for 30 min. Mitochondrial pellet and supernatant (Sup.) collected by centrifugation were analyzed for Bax by Western blotting. Bax release into supernatant indicates a loose association of Bax with mitochondria in normal cells. B, Bax<sup>−/−</sup> and Bax<sup>+/−</sup> HCT116 cells were treated with 2.5 μM CCCP for 7.5 h at 37 °C in Krebs-Ringer buffer. Following incubation of HCT116 cells with CCCP, membrane fractions were obtained and cross-linked with EGS as described under “Experimental Procedures,” and analyzed for Bax (lanes 1–6) or Bak (lanes 7–12) by Western blotting under reducing conditions. A typical blot from four independent experiments is shown. Prolonged exposure to CCCP resulted in the formation of oligomeric Bax in the membrane fraction as slow moving complexes (lanes 11 and 12). Similarly, slow moving Bak dimers appeared after ATP depletion in parallel with Bak oligomers (lanes 5 and 6). Note that there is a significant increase in Bak dimers only in ATP-depleted Bax<sup>+/−</sup> cells but not Bax<sup>−/−</sup> cells. The identities of slow moving bands intermediate in size between monomer and dimer in control cells (lanes 1 and 4) and corresponding to trimers in both ATP-depleted Bax<sup>−/−</sup> and Bax<sup>+/−</sup> cells (lanes 2, 3, 5, and 6) are not known. C, cytosolic digitonin extracts of Bax<sup>−/−</sup> and Bax<sup>+/−</sup> colon cancer cells after ATP depletion (7.5 h CCCP) were analyzed for released cytochrome c (Cyt.c in cytosol) by Western blotting.

gest that Bax is required to induce Bak reorganization and cytochrome c release during hypoxia or ATP depletion.

**DISCUSSION**

The release of cytochrome c from mitochondria is a crucial step in apoptotic signaling through the activation of caspases (30–32). Several studies point to a major role for Bax in cytochrome c release based on its ability to form channels in artificial lipid membranes (33) and large oligomeric complexes in the mitochondrial outer membrane (16–18). Although the formation of transmembrane channels by Bax oligomers is a likely explanation, the question of how Bax triggers cytochrome c release after its translocation to mitochondria continues to be debated. Here, we demonstrate that following Bax redistribution from the cytosol to mitochondria, formation of Bax oligomers in the mitochondrial outer membrane is also accompanied by reorganization of resident Bak molecules to homo-oligomerize. Analysis of the molecular sizes of Bax and Bak oligomers by cross-linking and SDS-PAGE indicated that both Bax and Bak oligomers are largely if not exclusively homogeneous. Our results show also that these homo-oligomeric complexes of both Bak and Bax co-precipitated with each other during immunoprecipitation.

Unlike Bax, Bak was found to be constitutively present in the mitochondria of RPTC (Fig. 1A, panel 1), in the form of large complexes by gel filtration (Fig. 3A) and chemical cross-linking (Fig. 1C). After hypoxia or ATP depletion, both Bax and Bak were co-localized in mitochondria (Fig. 1A, panel 2). Following translocation of Bax to mitochondria, Bak reorganized to form smaller oligomeric complexes that are absent in normal cells (Fig. 1, C and D). The molecular weights of Bax and Bak oligomers indicated that these complexes are homogeneous and are therefore homo-oligomers (Fig. 1D). By analogy to mechanisms that lead to pore formation by bacterial toxins, conformational changes may occur in Bak that lead to oligomerization following membrane insertion (34, 35). The observation that both Bax and Bak exhibit conformational changes during hypoxia or ATP depletion raises the question whether these two proteins are causally linked to the release of apoptogenic cytochrome c and Smac proteins from mitochondria. Based on previous reports that Bak oligomerization is primarily mediated by Bid, we explored the role of Bid in hypoxia or ATP depletion-induced apoptosis. Previous studies have shown that death stimuli by Fas ligand or tumor necrosis factor-α activate caspase-8 to cleave Bid, a BH3-only protein, to a truncated form (t-Bid) that is targeted to mitochondria, inducing Bak to oligomerize (21). However, in both RPTC and HeLa cells, Bid did not undergo either proteolytic cleavage or mitochondrial translocation during ATP depletion by CCCP or hypoxia. Moreover, failure to prevent Bax translocation and cytochrome c release from mitochondria of ATP-depleted cells by z-VAD (24, 36), a broad spectrum caspase inhibitor, is also indicative of the non-involvement of t-Bid. Therefore, it seems likely that Bax triggers a conformational change in Bak to homo-oligomerize following mitochondrial insertion.

Protein complex formation in mitochondrial membranes was also analyzed by size sieving chromatography. We chose CHAPS to solubilize membrane proteins because of its smaller aggregation number and its inability to induce conformational change in the Bcl-2 family of proteins (16, 29). Although co-purification of proteins in fractions separated by gel filtration does not necessarily mean interactions between the proteins, the converse should be true. That is, proteins with tight interactions between them should co-purify. In normal cells, both Bax and Bak have distinct elution profiles after gel filtration. The broader elution profile (43–5000 kDa) of Bak in normal cells (Fig. 3) suggests possible association of Bak with a protein or protein complex in the mitochondrial outer membrane. In contrast, the elution profile of Bax, which is cytosolic in normal cells, is sharper and indicative of its monomeric nature (Fig. 3). However, the elution profiles of both Bak (43–1340 kDa) and Bax (25–1340 kDa) were found to overlap significantly in ATP-depleted cells (Fig. 3). Our immunoelectron microscopy data on translocated Bax in mitochondria of ATP-depleted cells are in agreement with results reported by Youle’s group (37) in terms of the clustering of Bax on mitochondrial surfaces. However, we did not detect large clusters of Bax molecules outside...
the mitochondria in cells affected by ATP depletion-induced apoptosis (Fig. 3, panel 2). The immuno-EM findings are also consistent with our previous studies (16, 24) and current results showing Bax translocation, oligomerization, and cytochrome c release.

By several types of analysis including cross-linking, gel filtration, and immunoprecipitation, our results have further clarified the inhibitory effect of Bcl-2 expression on the deleterious effects of pro-apoptotic Bax and Bak. Bcl-2 prevented the relatively close association of Bax and Bak molecules with themselves to form chemically cross-linkable homo-oligomers, as well as the possibly looser association between these two different species of homo-oligomers to form larger coimmunoprecipitable complexes that could not be stabilized by cross-linking. More Bax and Bak were precipitated by their respective antibodies from ATP-depleted cells than from normal cells (Fig. 5). This can be attributed to increased antibody recognition of these antigens caused by unfolding of the COOH terminus of Bak and translocation of the protein (13, 29), and dissociation of Bak from large Bak containing complexes of otherwise unknown composition, native to mitochondrial membranes (Fig. 4). Relevant to the formulation of strategies to address still unresolved questions regarding how Bcl-2 protects cells, expression of this protein prevented neither the ATP depletion-induced mitochondrial translocation of Bax (16, 24), nor the associated dissolution of large Bak containing complexes (Figs. 4 and 5). However, Bcl-2 did prevent or markedly inhibited Bax and Bak homo-oligomerization and cross-immunoprecipitation of Bax and Bak that could otherwise be demonstrated easily in the absence of Bcl-2 expression (Figs. 4 and 5). The molecular interactions that underlie these remarkable results will need close attention in future studies.

Co-immunoprecipitation of Bax oligomers with Bak antibodies and Bak oligomers with Bax antibodies (Fig. 5) and the nonoverlapping nature of Bax and Bak ladders after co-precipitation (Fig. 5B) together suggest that these different homo-oligomeric species interact in the membrane. Interestingly, dissociation and reassociation studies have thrown light on the nature of interaction between Bax and Bak. Regardless of treatment with SDS prior to renaturation and cross-immunoprecipitation of cross-linked membranes, Bax as well as Bak containing oligomers (ladders) were largely, if not exclusively, homogeneous, i.e. they were homo-oligomeric. This suggests that molecular interactions between individual monomers in either Bax or Bak ladders were close, to the extent that they could be stabilized by cross-linking. The failure to cross-link proteins does not necessarily mean they are not bound to each other. Steric factors related to the chemical nature of the cross-
linkers and/or protein interactions are involved in cross-linking of proteins, and negative results need to be interpreted with caution. This was addressed by using a nonspecific heterobifunctional cross-linker SANPAH (N-succinimidy1-6-[4-azido-2'-nitrophenylelimino] hexanoate), which cross-links proteins through an amine reactive and a photostable nitrene that reacts nonspecifically with any atom within the reach of the spacer arm, and we obtained predominantly homo-oligomers (16). On the other hand, interactions between Bax and Bak must have been relatively loose, because they permitted coinmunoprecipitation, but could not be stabilized by cross-linking to any significant extent (for the exception see Fig. 6B, lane 6). If Bax-Bak interactions had been tight to the degree that both molecules contributed to the formation of hetero-oligomeric complexes (Bax-Bak pore), Bax and Bak ladders resolved by SDS-PAGE should have shown overlapping patterns. Obviously, this was not the case.

Our results show that trimers and higher order cross-linked oligomers of Bax reassociate with Bak during CHAPS renaturation after SDS denaturation to a much greater extent than do Bax-Bax dimers or Bax monomers (Fig. 6B, lane 6). The appearance of Bax-Bak heterodimers only as a minor component of these reassociated complexes (Fig. 6B, lane 6) further reinforces the concept that pores in the membrane are mainly constituted of homooligomeric Bax and Bak. Considered in their entirety, our results suggest that interaction between Bax and Bak takes place after Bax has undergone conformational change to oligomerize. Therefore, Bax oligomerization probably precedes Bak reorganization and oligomerization.

Gene knockout studies with Bax, Bak, and Bax plus Bak have indicated that cells with single gene knockout are capable of releasing cytochrome c, but those with double knockout are not (10, 25). Indeed, mice deficient of both Bax and Bak died normally and reproduced normally (9), whereas Bax knockout mice did not (10, 25). Indeed, mice deficient of both Bax and Bak alone not only failed to undergo reorganization but also failed to induce the release of cytochrome c upon ATP depletion (Figs. 8, B and D and 7C). On the other hand, homo-oligomerization of Bax or cytochrome c release does not require Bak (Fig. 8C). However, optimal Bak translocation does seem to need the presence of Bak (Fig. 8A). Whether this should be attributed to Bak alone or a Bak associated common target for Bax and Bak needs to be determined.

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