

Nonionic Detergents Induce Dimerization among Members of the Bcl-2 Family*

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Yi-Te Hsu and Richard J. Youle‡

From the Biochemistry Section, Surgical Neurology Branch, NINDS, National Institutes of Health, Bethesda, Maryland 20892

Members of the Bcl-2 family (including Bcl-2, Bcl-X_L, and Bax) play key roles in the regulation of apoptosis. These proteins are believed to be membrane-associated and have been proposed to regulate apoptosis through both homodimerization and heterodimerization. We have found that whereas Bcl-2 is predominantly membrane-associated as previously reported, significant amounts of Bcl-X_L and most of the Bax proteins are not membrane-associated and thus appear in the cytosolic fraction of thymocyte and splenocyte extracts. This finding allows the study of the dimerization properties and conformation of these proteins in the absence of detergent perturbation. For this analysis, we have produced monoclonal antibodies that are specific for known epitopes of Bax, Bcl-2, and Bcl-X_L. An antibody to an N-terminal epitope (α uBax 6A7) between amino acids 12 and 24 fails to bind the soluble cytosolic form of Bax, indicating that this epitope is normally buried. Nonionic detergents alter the Bax conformation to expose this epitope. In the presence of nonionic detergent, the 6A7 antibody avidly binds the monomeric form of Bax, but not Bax complexed with either Bcl-X_L or Bcl-2. In contrast, a monoclonal antibody to an adjacent epitope of Bax (α mBax 5B7) within amino acids 3–16 binds the soluble and detergent-altered forms of Bax and also binds the Bax-Bcl-X_L or the Bax-Bcl-2 complex. Surprisingly, in the absence of detergent Bax fails to form homodimers or heterodimers with Bcl-X_L. These results demonstrate a novel conformational state of members of the Bcl-2 family under a physiological condition that is distinct from the detergent-altered state that forms dimers and is currently believed to regulate apoptosis.

Bcl-2, Bax, and Bcl-X_L are members of a family of proteins that regulate apoptosis. The gene encoding Bcl-2 was first identified at the chromosomal translocation point t(14;18) in human B cell follicular lymphoma (1–3). Overexpression of Bcl-2 enhanced cell survival by suppressing apoptosis in a number of cells subjected to a wide range of apoptosis-inducing stimuli, including nerve growth factor withdrawal, radiation, and chemotherapeutic agents (for reviews, see Refs. 4–7). The gene encoding Bcl-X_L was first identified by homology cloning using the *bcl-2* gene as a probe (8). Like Bcl-2, overexpression of Bcl-X_L inhibited apoptosis that was induced by a variety of agents (9). Finally, Bax was identified as a protein that co-

immunoprecipitated with Bcl-2 (10). In contrast to Bcl-2 and Bcl-X_L, overexpression of Bax accelerated cell death (10).

By immunoprecipitation and yeast two-hybrid select studies, it appears that Bax may form homodimers or heterodimers with either Bcl-2 or Bcl-X_L (10–12). Formation of Bax homodimers has been proposed to promote cell death, and this could be blocked by Bax heterodimerization with Bcl-2 or Bcl-X_L. Based on Bax overexpression and dimerization studies, it has been postulated that the relative ratio of Bax homodimers to heterodimers may serve as a key sensory switch that dictates the initiation of apoptosis (10, 12, 13).

Dimerizations between Bax, Bcl-2, and Bcl-X_L are believed to be mediated through three highly conserved regions within each protein known as the BH1, BH2, and BH3 domains (14, 15). BH1 and BH2 domains are required for Bcl-2 and Bcl-X_L to heterodimerize with Bax. Mutations within these domains disrupt their heterodimerizations with Bax and abrogate their abilities to prevent apoptosis (12, 14). Furthermore, the BH3 domain of Bax has been shown to be essential for both its homodimerization and heterodimerization with either Bcl-2 or Bcl-X_L (15).

Bcl-2, Bax, and Bcl-X_L have a hydrophobic segment at their respective C-terminal ends (8, 10, 16). Hydrophathy plots predict that these segments may serve as membrane anchors. Subcellular fractionation studies have shown Bcl-2 to be localized to the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes (17, 18). Recently, immunocytochemical studies have localized Bcl-X_L to outer membranes of mitochondria (19). Bax has been suggested to co-localize within the same subcellular compartments as Bcl-2 (10) through its ability to dimerize with Bcl-2.

In this paper, we describe the generation of several epitope-specific monoclonal antibodies against Bcl-2, Bax, and Bcl-X_L. Using these antibodies, we probed the subcellular localization of Bax, Bcl-2, and Bcl-X_L in normal murine thymocytes and splenocytes and studied the conformational dependence of dimerization among members of the Bcl-2 family.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptides were purchased from Peptide Technologies Corp. Imject® maleimide-activated keyhole limpet hemocyanin (KLH)¹ was obtained from Pierce. pcDNA3 plasmid was from Invitrogen. PEG 4000, HAT medium (hypoxanthine/aminopterin/thymidine medium), and LipofectAMINE were from Life Technologies, Inc. NS-1 and HeLa cells were from the ATCC. Iscove's medium was from Biofluids, Inc. Hamster anti-murine Bcl-2 monoclonal antibody 3F11 and rabbit anti-calnexin polyclonal antibodies were from PharMingen and StressGen Biotech Corp., respectively. Sheep anti-mouse and donkey anti-rabbit immunoglobulin peroxidase conjugates and ECL Western blotting detection kit were purchased from Amersham Corp. Goat anti-hamster immunoglobulin peroxidase conjugate was from Kirkegaard and Perry Laboratories. Human thymic tissues were generous gifts

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‡ To whom correspondence should be addressed: Biochemistry Section, Surgical Neurology Branch, NINDS, National Institutes of Health, 10 Center Dr., Rm. 5D-37, Bethesda, MD 20892. Tel.: 301-496-6628; Fax: 301-402-0380; E-mail: youle@helix.nih.gov.

¹ The abbreviations used are: KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis.

from the Children's National Medical Center, Washington, D. C. All other reagents were obtained from Sigma.

Generation of Monoclonal Antibodies—Peptides corresponding to internal sequences of Bax, Bcl-2, and Bcl-X_L were conjugated to maleimide-activated KLH through the cysteine residue according to the protocol provided by the manufacturer. Anti-Bax monoclonal antibodies were generated by immunizing mice with KLH conjugated to peptides corresponding to amino acids 3–16 of murine Bax (CGSGEQLGSGGPTSS), amino acids 3–16 of human Bax (CGSGEQPRGGGPTSS), and amino acids 12–24 shared by Bax from both species (CGPTSSEQIMKTGA). Anti-murine Bcl-2 antibody was generated by immunizing mice with a KLH-conjugated peptide (amino acids 61–76) of murine Bcl-2 (CVHREMAARTSPLRPLV). Anti-Bcl-X_L antibody was generated by immunizing mice with a KLH-conjugated peptide having a sequence corresponding to amino acids 3–14 shared by both murine and human Bcl-X_L (CQSNRELVDVFLS). Splenocytes from immunoreactive mice were fused by PEG 4000 to murine NS-1 myeloma cells and selected with HAT medium (20). The anti-murine, human, and species-independent Bax antibodies were designated as α mBax 5B7, α hBax 2D2 (or 1F6), and α uBax 6A7, respectively. The anti-murine Bcl-2 antibody was designated as α mBcl-2 10C4. The anti-species-independent Bcl-X_L antibody was designated as α uBcl-X_L 2H12.

Transient Transfection of Murine Bax in HeLa Cells—Murine Bax cDNA (a kind gift of Dr. Stanley Korsmeyer) was cloned into pcDNA3. Eight plates (100 \times 20 mm) of HeLa cells at 80–90% confluency were each transfected with 16 μ g of mBax/pcDNA3 plasmid using the LipofectAMINE (128 μ g/plate) transfection protocol described in the product instructional guide. The cells were harvested at 36 h post-transfection, and soluble extract was prepared as described below.

Subcellular Localization of Bax, Bcl-2, and Bcl-X_L—Murine thymocytes and splenocytes were dispersed from their tissues in the presence of Iscove's medium. The cells were spun down, washed once in Iscove's medium, and resuspended in 70 ml of lysis buffer (10 mM Hepes, pH 7.4, 38 mM NaCl, 25 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin) at a cell density of 5×10^7 /ml. The hypotonic lysate was Dounce-homogenized. The homogenate was spun at 900 \times g to pellet the nuclei using a Sorvall SA-600 rotor. The postnuclear supernatant was then spun at 130,000 \times g in a Beckman Ti 45 rotor to pellet the membranes. Both the crude membranes and the nuclear pellet were resuspended in 70 ml of lysis buffer. The extent of cross-contamination between the soluble protein and the crude membrane preparations was assessed by assaying for either the cytosolic enzyme marker lactate dehydrogenase (21) or the mitochondrial enzyme marker cytochrome *c* oxidase (22). Anti-calnexin polyclonal antibody was used in Western blotting to determine the level of endoplasmic reticulum membranes in the cytosolic extract. The relative levels of Bax, Bcl-2, and Bcl-X_L in the cytosolic, crude membrane, and nuclear fractions were analyzed by Western blotting with the corresponding antibodies.

For the study of Bax homodimerization, human and murine thymocytes were hypotonically lysed at a cell density of 1×10^8 /ml, whereas HeLa cells transfected with mouse Bax were lysed at a cell density of 5×10^6 /ml. Soluble proteins from these cells were prepared as described above. The soluble extracts were used for the immunoprecipitation studies as described below.

Immunoprecipitation of Bax—Monoclonal antibodies α mBax 5B7, α hBax 1F6, and α uBax 6A7 were purified from ascites fluids by ammonium sulfate precipitation and DEAE fractionation (23, 24). The purified antibodies were immobilized onto CNBr-activated Sepharose 4B at 2.5 mg of protein/ml of packed beads (23, 24). For the immunoprecipitation of Bax from detergent-solubilized samples, murine splenocytes or thymocytes were solubilized in 10 ml of 10 mM Hepes, pH 7.4, 150 mM NaCl, and 1% Triton X-100 or 0.2% Nonidet P-40 at a cell density of 5×10^7 /ml in the presence of proteolytic inhibitors (25 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin). The lysate was spun at 14,000 rpm in a Sorvall SA 600 rotor for 15 min to pellet the nuclei and the unsolubilized material. 4.5 ml of the detergent-solubilized lysate was then mixed with 150 μ l of the α mBax 5B7 or α uBax 6A7 antibody beads and allowed to incubate for 2 h at 4 °C. The beads were then washed twice with 10 ml of solubilization buffer containing either 0.2% Triton X-100 or 0.2% Nonidet P-40. The bound proteins were then eluted from the beads by the addition of 180 μ l of 0.1 M acetic acid containing either 0.2% Triton X-100 or Nonidet P-40. The acid eluants were neutralized by the addition of 30 μ l of 1 M Tris, pH 8.0.

For the immunoprecipitation of murine Bax from the cytosolic extract of murine thymocytes, the NaCl concentration of the extract was adjusted to 150 mM, and the extract was then filtered through a

0.45- μ m filter (Millipore Corp.). 4.5 ml of the soluble extract was mixed with 150 μ l of α mBax 5B7 or α uBax 6A7 antibody beads either in the absence or presence of 0.2% Triton X-100 or Nonidet P-40 and allowed to incubate for 2 h. The unbound proteins were removed by washing the beads twice with 10 ml of 10 mM Hepes, pH 7.4, and 150 mM NaCl or with the same buffer containing either 0.2% Triton X-100 or Nonidet P-40. The bound proteins were then eluted from the beads with 180 μ l of 0.1 M acetic acid containing either 0.2% Triton X-100 or Nonidet P-40. The acid eluants were neutralized with 30 μ l of 1 M Tris, pH 8.0.

For Bax homodimerization studies, 4.5 ml of the mixed human and murine cytosolic extracts (with the NaCl concentration adjusted to 150 mM) that had been filtered were allowed to incubate at 4 °C for 1 h either in the absence or presence of 0.2% Triton X-100 or Nonidet P-40. The three different extracts were then mixed with 150 μ l of α hBax 1F6 antibody beads, and immunoprecipitation was carried out as described above. Immunoprecipitation of murine Bax from transfected HeLa cells was carried out by mixing 150 μ l of α mBax 5B7 antibody beads with 4.5 ml of the filtered soluble extract from transfected HeLa cells either in the absence or presence of 0.2% Triton X-100 or Nonidet P-40. The washing and elution steps were performed as described above.

SDS-PAGE and Western Blotting—SDS-polyacrylamide gel electrophoresis was carried out on 12% polyacrylamide gels according to the method of Laemmli (25). 23 μ l of the samples from subcellular fractionation and immunoprecipitation studies were analyzed by SDS-PAGE. The gels were either stained with Coomassie Blue or electroblotted onto Immobilon membranes (20). For immunoblotting analysis, the blots were blocked in phosphate-buffered saline, 0.05% Tween 20 containing 5% nonfat milk or fetal bovine serum and incubated with either α hBax 2D2 (1:10 diluted culture fluid), α mBax 5B7 (1:10 diluted culture fluid), α uBcl-X_L 2H12 (1:10 diluted culture fluid), α mBcl-2 10C4 (1:10 diluted culture fluid), hamster anti-murine Bcl-2 monoclonal antibody 3F11 (1 μ g/ml), or rabbit anti-calnexin polyclonal antibody (1:1000 dilution) diluted in the blocking buffer for 45 min. The blots were then washed in phosphate-buffered saline, 0.05% Tween 20 and incubated in blocking buffer containing either 1:5000 diluted sheep anti-mouse, donkey anti-rabbit, or goat anti-hamster immunoglobulin peroxidase for an additional 30 min. The blots were washed again in phosphate-buffered saline, 0.05% Tween 20 and visualized with ECL Western blotting detection kit.

RESULTS

Generation of Monoclonal Antibodies—Several monoclonal antibodies were raised against Bax, Bcl-2, and Bcl-X_L for the purpose of Western blotting detection and immunoprecipitation analysis. The α hBax 2D2 and hBax 1F6 monoclonal antibodies were directed against an N-terminal peptide (amino acids 3–16) of human Bax, and they do not cross-react with either murine or rat Bax. On the other hand, the α mBax 5B7 monoclonal antibody was raised against an N-terminal peptide (amino acids 3–16) of murine Bax and is specific for mouse. The α uBax 6A7 monoclonal antibody was produced against a peptide sequence (amino acids 12–24) common to human, murine, and rat Bax, and this antibody binds to Bax from all three species. The α mBcl-2 10C4 monoclonal antibody was raised against a murine Bcl-2 peptide (amino acids 61–76), and this antibody cross-reacts with rat Bcl-2. Lastly, the α uBcl-X_L 2H12 monoclonal antibody was generated against an N-terminal peptide (amino acids 3–14) common to human and murine Bcl-X_L, and this antibody binds to human, murine, and rat Bcl-X_L.

Subcellular Localization of Bax, Bcl-2, and Bcl-X_L—We have recently found a differential compartmentalization of Bax, Bcl-2, and Bcl-X_L (30). To further confirm this finding, murine thymocytes and splenocytes were hypotonically lysed and Dounce-homogenized. The cell lysates were separated into soluble, crude membrane, and nuclear fractions by differential centrifugation. As shown on the Coomassie Blue-stained SDS-polyacrylamide gel (Fig. 1), the total proteins from these three different subcellular compartments (*lanes c, d, and e*) have distinct banding patterns in both thymus and spleen. Contamination from mitochondria and endoplasmic reticulum was not detected in the soluble protein fractions as determined by cy-

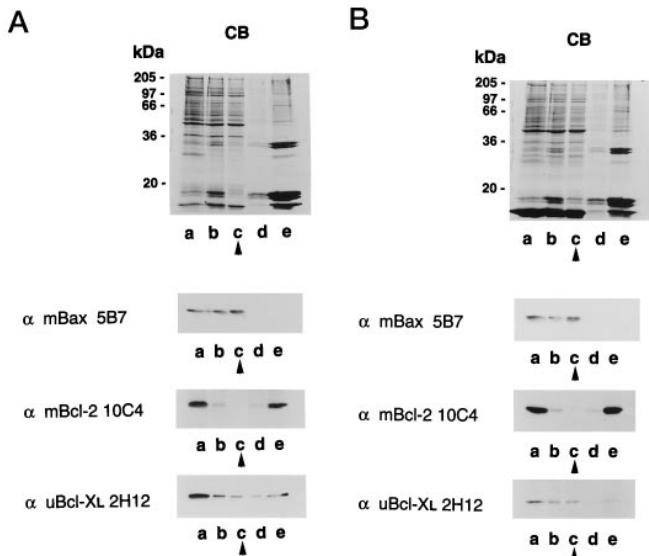


FIG. 1. Solubility of Bax, Bcl-2, and Bcl-X_L in murine thymocytes (A) and splenocytes (B). Murine thymocytes and splenocytes were hypotonically lysed and Dounce-homogenized. Low speed post-nuclear supernatant (lanes b), high speed supernatant (lanes c, arrow), high speed membrane pellet (lanes d), and nuclear pellet (lanes e) were prepared by differential centrifugation. Lanes a are Triton X-100-solubilized thymocytes or splenocytes. The protein samples were analyzed by both SDS-polyacrylamide gel stained with Coomassie Blue (CB) and Western blotting with α mBax 5B7, α mBcl-2 10C4, and α uBcl-X_L 2H12 monoclonal antibodies.

tochrome *c* oxidase activity assay and Western blotting analysis with an anti-calnexin polyclonal antibody, respectively (data not shown). Western blotting analyses of these samples revealed Bax as a predominantly soluble protein in both thymocytes (Fig. 1A, lane c, arrow) and splenocytes (Fig. 1B, lane c, arrow). Bcl-2, on the other hand, was found in both crude membrane and nuclear fractions but was not detected in the soluble fraction (Fig. 1, A and B, lanes d and e). Finally, Bcl-X_L was found to be present in all three compartments of thymocytes (Fig. 1A, lanes c, d, and e) and in soluble and nuclear fractions of splenocytes (Fig. 1B, lanes c and e). These results indicate that Bax, Bcl-2, and Bcl-X_L are differentially compartmentalized with Bax being predominantly soluble, Bcl-2 being exclusively membrane-bound, and Bcl-X_L being present in both soluble and membrane-bound forms.

Effect of Detergent on the Heterodimerization of Bax—The differential subcellular localization of Bax and Bcl-2 in both thymocytes and splenocytes raises uncertainty as to whether the formation of Bax:Bcl-2 heterodimer complexes exist in healthy living cells. The finding of the soluble cytosolic localization of Bax and a significant fraction of Bcl-X_L (Fig. 1) eliminates the necessity of detergent solubilization to analyze Bax heterodimer formation. Therefore, immunoprecipitation of Bax from the cytosolic extract of murine thymocytes in the absence of detergent was compared with that in the presence of the detergents Triton X-100 or Nonidet P-40. These nonionic detergents do not disrupt Bax heterodimer formation. Anti-Bax monoclonal antibody 5B7 was used to immunoprecipitate Bax. The immunoprecipitated samples were analyzed by Western blotting with α uBcl-X_L 2H12 monoclonal antibody for the detection of murine Bcl-X_L. In the absence of detergent, immunoprecipitation of Bax by the α mBax 5B7 antibody failed to co-precipitate Bcl-X_L (Fig. 2, lower left panel, lane c). However, if either Triton X-100 or Nonidet P-40 was present during the immunoprecipitation, a significant amount of Bcl-X_L was co-precipitated with Bax (Fig. 2, lower middle and right panels, lanes c). Immunoprecipitation analysis has also been carried

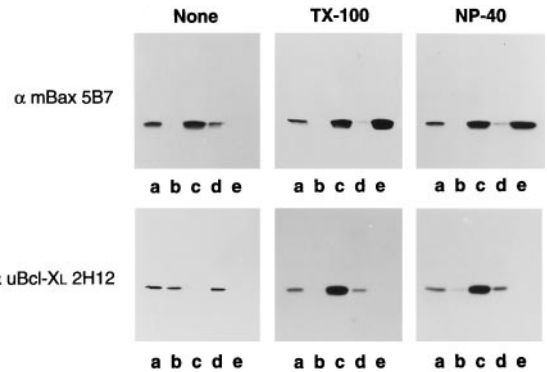


FIG. 2. Immunoprecipitation of Bax from the cytosolic extract of murine thymocytes in the absence or presence of detergent. High speed soluble extract of murine thymocytes without (left panels, lanes a) or with (middle panels, lanes a) the addition of Triton X-100 or Nonidet P-40 (right panels, lanes a) was immunoprecipitated with α mBax 5B7 (lanes c) or α uBax 6A7 (lanes e) antibody Sepharose beads. The lanes b and d represent the unbound proteins in the supernatant of lysates immunoprecipitated with α mBax 5B7 and α uBax 6A7 antibody Sepharose beads, respectively. The protein samples were analyzed by Western blotting with either α mBax 5B7 (upper panels) or α uBcl-X_L 2H12 (lower panels) monoclonal antibodies.

out using murine splenocyte cytosolic extract and has yielded similar detergent-dependent binding of Bax with Bcl-X_L (data not shown). Since there is no evidence of Bax association with Bcl-X_L in the absence of detergent, it appears that detergent induces a conformational change in Bax that facilitates the heterodimerization process. The lack of Bax/Bcl-X_L heterodimer formation in the absence of detergent together with the mutually exclusive localization of Bax and Bcl-2 stimulates a reconsideration of the possible roles that dimer formation may play in the regulation of apoptosis.

Detergent Exposure of an N-terminal Epitope Correlates with Heterodimer Formation—Immunoprecipitation of Bax from the cytosolic extract of murine thymocytes with α mBax 5B7 antibody was compared with that of the α uBax 6A7 antibody, which is an antibody whose epitope (amino acids 12–24) partially overlaps or is very near to that of the 5B7 antibody (amino acids 3–16). The immunoprecipitated samples were analyzed by Western blotting with α mBax 5B7 monoclonal antibody for the detection of murine Bax. Whereas the binding of murine Bax to α mBax 5B7 antibody occurred in the presence or absence of detergents (Fig. 2, upper panels, lanes c), the α uBax 6A7 antibody required the presence of either Triton X-100 or Nonidet P-40 detergent during immunoprecipitation to bind Bax (Fig. 2, upper panels, lanes e). A similar detergent-dependent binding of Bax to the α uBax 6A7 antibody was observed with murine splenocyte cytosolic extract (data not shown). Thus, the amino acid 12–24 segment in the N terminus of Bax is normally inaccessible to 6A7 antibody binding and becomes exposed in detergent. This is particularly interesting because the heterodimerization of Bax also depends on the presence of detergent (Fig. 2), suggesting that a conformational change exposing this N-terminal epitope region may be involved in heterodimer formation.

To test this hypothesis, we examined heterodimer formation by immunoprecipitation with the α uBax 6A7 antibody. In the presence of detergent, the α uBax 6A7 monoclonal antibody bound only to Bax species that were not associated with Bcl-X_L (Fig. 2, lower middle and right panels, lanes e in each panel). We further examined the competition between the 6A7 antibody binding to Bax and Bax heterodimer formation with Bcl-X_L and Bcl-2. Anti-Bax monoclonal antibodies α mBax 5B7 and α uBax 6A7 were used for the immunoprecipitation of Bax from the total cell lysates of murine thymocytes (Figs. 3A and

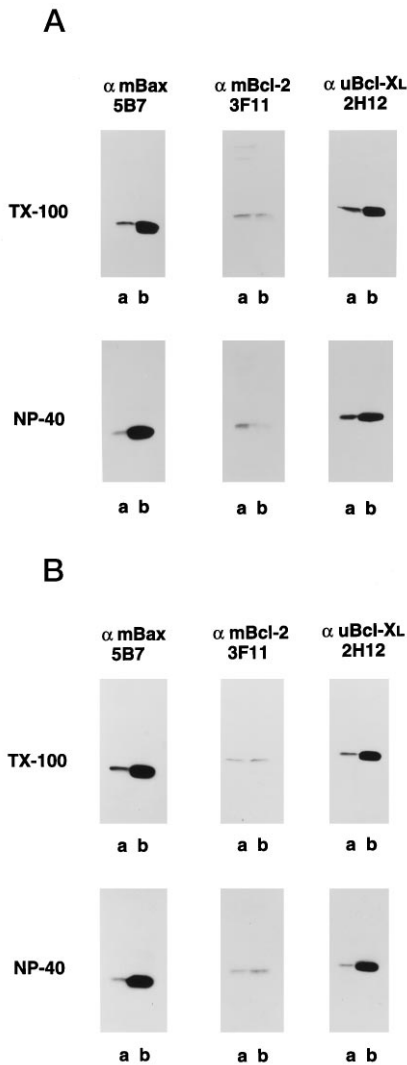


FIG. 3. Heterodimerization of Bax with Bcl-2 and Bcl-X_L. Murine thymocytes (A) and splenocytes (B) were solubilized in the presence of either Triton X-100 (upper panels) or Nonidet P-40 (lower panels). Bax was immunoprecipitated from the detergent-solubilized lysates with α mBax 5B7 antibody Sepharose beads. The presence of Bax and its associated Bcl-2 and Bcl-X_L in the total lysate (lanes a) and in the immunoprecipitated samples (lanes b) was detected by Western blotting analyses with α mBax 5B7 (left), hamster anti-murine Bcl-2 3F11 (middle), and α uBcl-X_L 2H12 (right) monoclonal antibodies.

4A) and splenocytes (Figs. 3B and 4B). Immunoprecipitations were carried out in the presence of either 1% Triton X-100 or 0.2% Nonidet P-40 (10). The immunoprecipitated samples were analyzed by Western blotting with either α mBax 5B7, hamster α mouse Bcl-2 3F11, or α uBcl-X_L 2H12 monoclonal antibodies. As shown in Fig. 3, immunoprecipitation with the α mBax 5B7 antibody from either thymocytes or splenocytes resulted in the co-precipitation of Bcl-2 and Bcl-X_L with Bax in the presence of either Triton X-100 or Nonidet P-40. The immunoprecipitation patterns with the α uBax 6A7 monoclonal antibody as shown in Fig. 4, however, appeared to be quite different. Neither Bcl-2 nor Bcl-X_L from thymocytes or splenocytes was found to associate with the α uBax 6A7 antibody-immunoprecipitated Bax in the presence of either Triton X-100 or Nonidet P-40. This suggests that the epitope for the 6A7 antibody (amino acids 12–24) may be in the vicinity of the dimerization domain of Bax so that the antibody directly competes with either Bcl-2 or Bcl-X_L for binding to Bax. This is interesting because the 6A7 antibody epitope partially overlaps or is adjacent to the 5B7 antibody

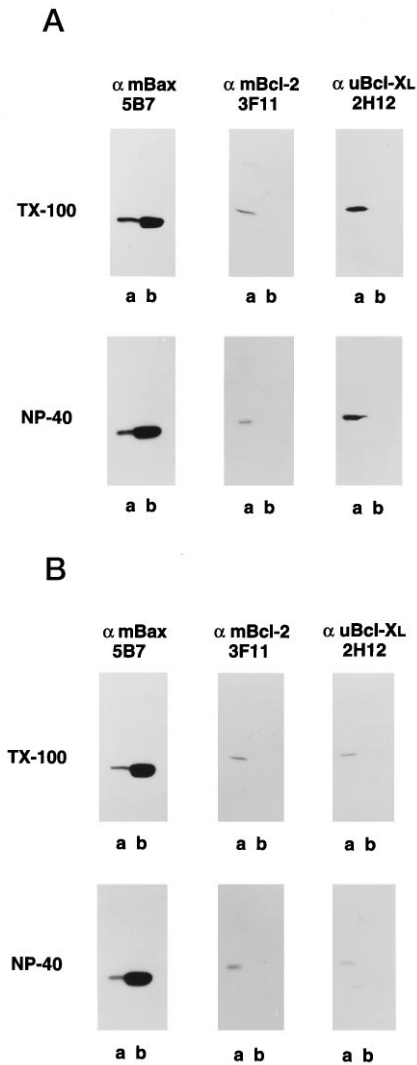


FIG. 4. Anti-Bax 6A7 monoclonal antibody recognizes only the heterodimerization-free state of Bax. Murine thymocytes (A) and splenocytes (B) were solubilized in either Triton X-100 (upper panels) or Nonidet P-40 (lower panels). Bax was immunoprecipitated from the detergent lysates (lanes a) by α uBax 6A7 antibody Sepharose beads. The absence of Bcl-2 and Bcl-X_L with the immunoprecipitated Bax samples (lanes b) was determined by Western blotting analyses with α mBax 5B7 (left), hamster anti-murine Bcl-2 3F11 (middle), and α uBcl-X_L 2H12 (right) monoclonal antibodies.

epitope (amino acids 3–16) and yet the latter antibody does not cross-compete with Bcl-2 and Bcl-X_L for binding to Bax.

Effect of Detergent on the Homodimerization of Bax—Bax has been previously reported to homodimerize in the presence of Nonidet P-40 (10). Considering that Bax did not heterodimerize with Bcl-X_L in the soluble detergent-free state, we examined whether Bax homodimerization required detergent. Bax was immunoprecipitated from a mixture of the soluble extracts of human and murine thymocytes either in the absence of detergent or in the presence of Triton X-100 or Nonidet P-40. Human Bax was immunoprecipitated from the mixture by α hBax 1F6 monoclonal antibody to determine whether human Bax can dimerize with murine Bax. This was made possible because the N-terminal epitope recognized by the anti-human Bax antibody 1F6 is distinct from murine Bax. The immunoprecipitated samples were analyzed by Western blotting with species-specific anti-human Bax 2D2 and anti-murine Bax 5B7 monoclonal antibodies (Fig. 5A). As revealed by Western blotting with α hBax 2D2 antibody, the affinity of anti-human Bax antibody 1F6 to Bax was not affected by detergent as evidenced by the

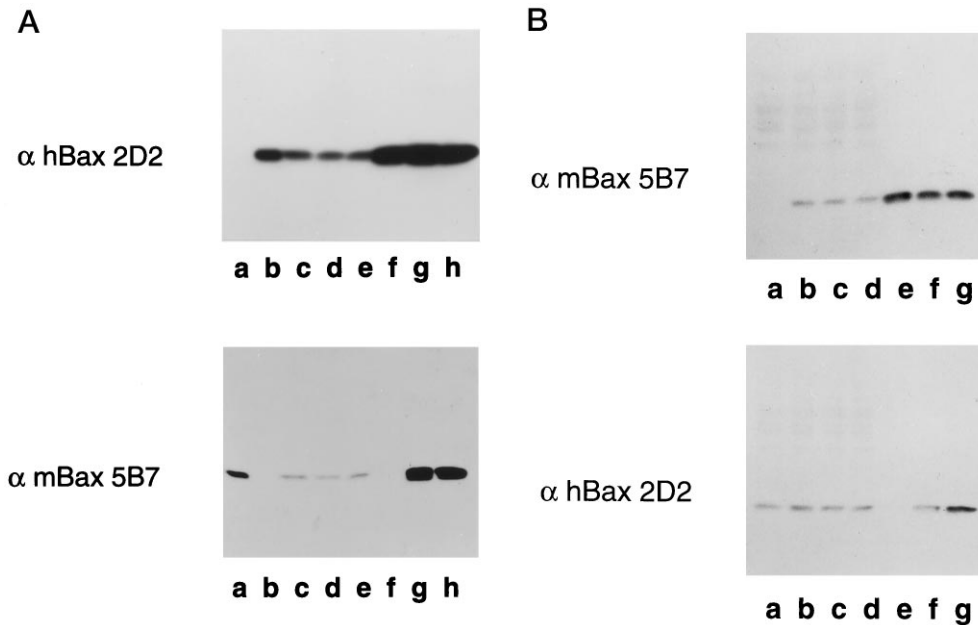


FIG. 5. **Detergent-dependent homodimerization of Bax.** A, combined soluble extracts from human and murine thymocytes either in the absence of detergent (lanes c) or in the presence of Triton X-100 (lanes d) or Nonidet P-40 (lanes e) were immunoprecipitated with α hBax 1F6 antibody Sepharose beads. Immunoprecipitation was carried out either in the absence of detergent (lanes f) or in the presence of Triton X-100 (lanes g) or Nonidet P-40 (lanes h). Lanes a and b are the murine and human thymocyte soluble extracts, respectively. The protein samples were analyzed by Western blotting with either α hBax 2D2 (upper panel) or α mBax 5B7 (lower panel) monoclonal antibodies. B, soluble extracts from HeLa cells transiently transfected with murine Bax were immunoprecipitated with α mBax 5B7 antibody Sepharose beads. Immunoprecipitation was carried out either in the absence of detergent (lanes e) or in the presence of Triton X-100 (lanes f) or Nonidet P-40 (lanes g). Lanes a are the soluble extract from untransfected HeLa cells, whereas lanes b, c, and d are the soluble extracts from transfected HeLa cells either in the absence of detergent or in the presence of Triton X-100 or Nonidet P-40, respectively. The protein samples were analyzed by Western blotting with either α mBax 5B7 (upper panel) or α hBax 2D2 (lower panel) monoclonal antibodies.

equal amounts of human Bax being immunoprecipitated in the absence and presence of detergent (Fig. 5A, top, lanes f–h). Homodimerization of human Bax to murine Bax as determined by Western blotting with α mBax 5B7 antibody occurred readily in the presence of detergent (Fig. 5A, bottom, lanes g and h). However, in the absence of detergent dimerization between human and murine Bax failed to occur (Fig. 5A, bottom, lane f).

Nevertheless, it is possible that the endogenous Bax present in murine and human thymocytes is already in a tight homodimer state, and that the addition of detergent merely allowed the exchange of partners. To examine this possibility, we transiently expressed murine Bax in HeLa, a human cervical carcinoma cell line that expresses endogenous human Bax. Immunoprecipitation of murine Bax was carried out from the soluble extract of transfected HeLa cells by α mBax 5B7 antibody beads either in the absence of detergent or in the presence of detergent Triton X-100 or Nonidet P-40. This allows us to determine whether murine and human Bax forms homodimers when co-expressed or whether the formation of homodimers is induced artificially by detergents. As shown by Western blotting analysis with α mBax 5B7 antibody, murine Bax is expressed in transfected HeLa cells (Fig. 5B, top, lanes b–d). The affinity of α mBax 5B7 antibody to Bax was again shown to be unaffected by detergent as evidenced by the equal amounts of murine Bax being immunoprecipitated in the absence and presence of detergent (Fig. 5B, top, lanes e–g). Western blotting analysis with α hBax 2D2 antibody revealed that whereas human Bax readily dimerizes with murine Bax in the presence of detergent Triton X-100 (Fig. 5B, bottom, lane f) or Nonidet P-40 (Fig. 5B, bottom, lane g), no dimerization was observed in the absence of detergent (Fig. 5B, bottom, lane e). These results suggest that Bax homodimerization, as was seen for Bax heterodimerization (Fig. 2), is a detergent-induced process.

DISCUSSION

Bcl-2, Bax, and Bcl-X_L are believed to be a group of membrane proteins that regulate apoptosis through *in vivo* dimerizations (10, 12, 13). In healthy cells, the formation of proapoptotic Bax homodimers is thought to be countermanded by Bax heterodimerization with pro-survival factors Bcl-2 or Bcl-X_L. Our finding that both Bax and Bcl-X_L are soluble (30) allowed us to explore the dimerization process without perturbing the protein structures with detergent. Surprisingly, in the absence of detergent Bcl-X_L failed to co-immunoprecipitate with Bax by the α mBax 5B7 antibody, whereas these two proteins readily co-immunoprecipitated in the presence of detergent. In addition, Bax homodimerization also was found to be dependent upon the presence of detergent. The differential localization of Bax and Bcl-2 suggests that these two proteins do not tightly associate in healthy thymocytes and splenocytes.

The above observations raised questions as to the extent to which Bax heterodimer and homodimer formation occur under physiological conditions. Fig. 6 proposes a model of the detergent-induced dimerization of Bax. The observations that Bax did not homodimerize or heterodimerize with Bcl-X_L in the absence of detergent indicate that the BH3 domain of Bax, which is crucial for Bax homodimerization and heterodimerization (15), may be inaccessible under physiological conditions. Immunoprecipitation studies with α uBax 6A7 antibody also indicate that the amino acid residues 12–24 of Bax are not exposed in the cytosol in the absence of detergent. The addition of detergent may thus cause a conformational change of Bax and reveal both the BH3 domain for Bax homo- and heterodimerization and the 6A7 epitope for antibody binding. Detergent is generally known to disrupt protein-protein interactions. However, in the case of Bax, it seems to promote heterodimeric and homodimeric complexes. At present, without

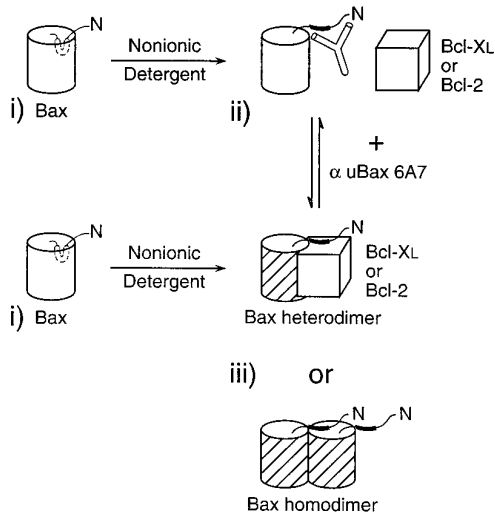


FIG. 6. Schematic representations of dimerizations between Bax, Bcl-2, and Bcl-X_L. Based on the immunoprecipitation results, Bax in its native conformation is likely to exist as a monomeric protein with its α 6A7 antibody epitope and BH3 dimerization domain inaccessible within the interior of the protein. Addition of nonionic detergent probably causes a conformational change in Bax (cross-hatched) and exposes the 6A7 antibody epitope and the BH3 dimerization domain. This allows the α uBax 6A7 antibody to bind Bax (ii), which in turn can inhibit the dimerization process. Bax in its detergent-induced conformation can also form either a heterodimer with Bcl-2 or Bcl-X_L or a homodimer with itself (iii). At present, it is not known whether detergent also causes similar conformational changes in Bcl-2 and Bcl-X_L to enable this dimerization process to take place.

the equivalent conformation-sensitive antibodies to Bcl-2 and Bcl-X_L, it is not yet known whether such detergent-induced conformational changes also occur in Bcl-2 and Bcl-X_L to allow for the exposure of BH1 and BH2 dimerization domains.

In addition to immunoprecipitation analyses, dimerization of Bax also has been observed in both the yeast two-hybrid select systems and the recombinant glutathione *S*-transferase-fusion protein binding studies (11, 12, 15, 26). In both cases, Bax was present as a fusion protein. It is possible that Bax, as part of a fusion protein, displayed an alternate conformation that is similar to the detergent state of this protein with the BH3 domain readily accessible for dimerization. Moreover, by site-directed mutagenesis analyses of Bcl-X_L and Bax, it was reported that heterodimerization of Bcl-X_L with Bax is not necessary for death-repressing activity (27, 28). Those results can be explained by our findings that soluble Bax and Bcl-X_L do not form dimers in the cytoplasm in the first place. Mutations described in these two studies appeared to have eliminated the "detergent-induced" Bax heterodimer formation.

The α uBax 6A7 antibody, which recognizes the detergent-induced exposure of the amino acid 12–24 epitope, also appears to compete with the heterodimeric partners of Bax. The α uBax 6A7 antibody-immunoprecipitated Bax was not associated with either Bcl-2 or Bcl-X_L. This suggests that the dimerization of Bcl-X_L and Bcl-2 to Bax may cover up the 6A7 antibody epitope on Bax. Alternatively, the binding of α uBax 6A7 antibody to Bax may block the heterodimerization of Bax to Bcl-2 and Bcl-X_L by steric hindrance. Thus, under detergent-solubilized conditions the epitope for the 6A7 antibody may be in close proximity to the BH3 dimerization domain of Bax (15) at the tertiary structural level. Recently, the N-terminal region of the Bax homolog Bcl-X_L has been also shown to be in proximity to its BH3 domain by x-ray crystallography (29).

Bax homodimers have been proposed to accelerate cell death, and this can be blocked by Bax heterodimerization with Bcl-2 and Bcl-X_L (10, 12). In our studies, we detected no Bax dimers

in the cytosol. It is possible, however, that the Bax molecules that migrate into membranes during apoptosis (30) may acquire the detergent-induced dimer-promoting state of Bax. Alternatively, it is possible that Bax, Bcl-2, and Bcl-X_L, which induce opposite effects, may be involved in novel binding with and regulation of other cellular proteins that may function in opposing biochemical pathways. Overexpression of either Bax or Bcl-2 and Bcl-X_L may then shift the equilibrium that favors either cell death or survival. If dimerizations of these proteins do not occur *in vivo*, then it would be crucial to determine whether these proteins compete for the binding of a common partner. Binding to a common partner could explain why mutations in BH1 and BH2 domains of Bcl-2 destroy its function. Resolving these issues will undoubtedly bring us closer to understanding the biochemical functions of members of the Bcl-2 family.

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