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Bax in Murine Thymus Is a Soluble Monomeric Protein That Displays Differential Detergent-induced Conformations*

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Bcl-2, Bcl-X_L, and Bax are members of the Bcl-2 family that play important roles in apoptosis regulation. These proteins are believed to be membrane-bound and to regulate apoptosis through formation of homo- and heterodimers. However, we recently found by subcellular fractionation that whereas Bcl-2 is predominantly a membrane protein as previously reported, Bax and a significant fraction of Bcl-X_L are soluble in thymocyte and splenocyte extracts. In addition, we have demonstrated that the ability of Bax to form dimers appears to be a detergent-induced phenomenon that coincides with a detergent-induced conformational change. We have further investigated the tertiary and quaternary states of Bax in the presence of various detergents. Detergents such as Triton X-100 and Triton X-114 readily enable Bax hetero- and homodimerization. However, other detergents such as polydocanol, W-1, octyl glucoside, dodecyl maltoside, Tween 20, and sodium cholate allow varying degrees of Bax hetero- and homodimerization. Detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (Chaps) and Brij 35 allow neither hetero- nor homodimer formation. Immunoprecipitation analysis with the conformation-sensitive antibody uBax 6A7 revealed that whereas Triton X-100 readily exposes the N-terminal Bax epitope (amino acid 13–19), only limited exposure of the epitope occurs in Triton X-114, polydocanol, dodecyl maltoside, and sodium cholate, and no exposure of this epitope was observed in W-1, Chaps, octyl glucoside, Tween 20, and Brij 35. Moreover, we could not detect any proteins associated with the cytosolic form of Bax based on immunopurification of this protein. Sephacryl S-100 gel filtration chromatography analysis of the cytosolic Bax indicated that this protein is monomeric and displays an apparent molecular mass of 25 kDa. Induction of apoptosis which causes the insertion of the soluble form of Bax into membranes did not result in appreciable Bax/Bcl-X_L, Bax/Bcl-2 or Bax/Bax dimer formation as determined by cross-linking studies. Further analysis of Bax after apoptosis induction by immunoprecipitation in the presence of Chaps also revealed no significant heterodimer formation. In conclusion, Bax displays several distinct states in different detergents that expose defined regions of the protein. In addition, these results suggest that mechanisms other than the simple dimerization among members of the Bcl-2 family may be required for the regulation of apoptosis.

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Apoptosis is a natural cell elimination process that occurs widely among multicellular organisms. Members of the Bcl-2 family including Bcl-2, Bcl-X_L, and Bax represent some of the most well known regulators of this process. Bax was first described as a pro-apoptotic protein that can bind and counteract the pro-survival function of Bcl-2 (1). Overexpression of Bax in transfected mammalian cells in many cases makes the cells more susceptible to apoptosis induced by external stimuli (for reviews see refs. 2 and 3), whereas only in some isolated cases it suppresses cell death (4, 5). Expression of Bax in fission yeast, however, has been reported to directly induce either growth arrest or cell death (6–10).

Like Bcl-2 and Bcl-X_L, Bax has three highly conserved regions known as BH1, BH2, and BH3 domains. The BH3 domain of Bax has been reported to be crucial for dimer formation (11). Based on yeast two-hybrid select systems and/or immunoprecipitation studies, it has been reported that Bax can form homodimers (1) or heterodimers with Bcl-2, Bcl-X_L, and Bid (1, 12–14). It can also form heterodimers with adenovirus-encoded Bcl-2 homolog E19 kD (15–17) and herpesvirus saimiri-encoded Bcl-2 homolog ORF16 (18) but not with a Bcl-2 homolog KS-bcl-2 encoded by herpesvirus 8 (19). In addition to these dimerization domains, Bax also has a predicted membrane spanning segment at its C-terminal end. In Bcl-2, this C-terminal hydrophobic region is responsible for anchoring this protein to membranes of various organelles including mitochondria, endoplasmic reticulum, and nuclei (20–22). Based on the presence of this hydrophobic region and the propensity of Bax to form heterodimers with Bcl-2, it was believed that Bax co-localizes with Bcl-2 in membranes. However, recently, several studies have shown that Bax is predominantly a soluble protein in thymocytes, splenocytes, and HL-60 promyelocytic leukemia cells (23, 24).

Functional analysis of Bax by knock-out studies indicate that this protein is essential for spermatogenesis (25, 26). In a double knock-out system, a deficiency in Bax prevents an increased cell death in the immature neurons of Bcl-X_L knock-out mice (27). Bax has been also implicated in anti-viral defense in promoting cell death in virally infected cells (28). In addition, Bax has been described as a tumor suppressor (29), and in certain cases of human colorectal cancer, frameshift mutations were found in the gene encoding Bax (30, 31).

Physiologically, Bax plays the role of sensitizing cells to apoptosis. However, little is known about the molecular basis by which Bax promotes cell death. One hypothesis, known as the dimer rheostat model, suggests that the formation of Bax homodimers promotes cell death, and in healthy living cells, the formation of Bax homodimers were prevented by Bax heterodimerization with the prosurvival factors Bcl-2 and Bcl-X_L (1, 32). However, several recent mutagenesis studies show that dimerization may not be essential for the regulation of apoptosis (33–35). Furthermore, we found that the cytosolic Bax in

murine thymocytes undergoes a detergent-induced conformational change that is associated with the propensity of this protein to form either homodimers or heterodimers with Bcl-2 and Bcl-X_L (24). In this paper we have explored the epitope exposure and dimerization of the cytosolic and membrane-bound Bax and identified several different conformational states of this protein in the presence of various types of detergents.

EXPERIMENTAL PROCEDURES

Materials—-Synthetic peptides were purchased from Peptide Technologies Corp. Imject maleimide-activated keyhole limpet hemocyanin was obtained from Pierce. PEG 4000, fetal bovine sera, and hypoxanthine/aminopterin/thymidine medium were from Life Technologies, Inc. Iscove's medium was from Biofluid. Disuccinimidyl glutarate (DSG)[1] and dithiobis(succinimidyl propionate) (DSP) cross-linkers were from Pierce. Fractogel EMD TMAE-650M and AF-heparin-650M beads were bought from EM Separations Technology and TosoHaas, respectively. SPOTs peptides were obtained from Genosys. Sheep anti-mouse immunoglobulin peroxidase conjugate and ECL Western blotting detection kit were purchased from Amersham Pharmacia Biotech. Bolton-Hunter reagent was from NEN Life Science Products. Immobilon membranes were from Millipore. All other reagents were obtained from Sigma.

Generation of Monoclonal Antibodies—New anti-Bax monoclonal antibodies were generated by immunizing mice with keyhole limpet hemocyanin conjugated to peptides corresponding to amino acids 3–16 of rat Bax (CGSGDHLGGGGPTSS) and amino acids 43–62 of mouse Bax (PELTLEQPPQDASTKKLSEC). Splenocytes from immunoreactive mice were fused by PEG 4000 to murine NS-1 myeloma cells and selected with hypoxanthine/aminopterin/thymidine medium (24, 36). The anti-rat and species-independent Bax antibodies were designated as α rBax 1D1 and α uBax 2C8, respectively.

Immunoprecipitation Analysis 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 (24). The purified antibodies were immobilized onto CNBr-activated Sepharose 4B at 2.5 mg of protein/ml packed beads (24, 37). For the detergent-dependent Bax heterodimerization and uBax 6A7 antibody binding studies, murine thymocytes were subjected to hypotonic lysis and Dounce homogenization at a cell density of 5×10^7/ml essentially as described previously (24). Soluble protein extracts prepared from high speed centrifugation ($130{,}000 \times g$) were adjusted to 150 mM NaCl either in the absence of detergent or in the presence of 0.2% Triton X-100, Triton X-114, polydocanol, W-1, Chaps, octyl glucoside, dodecyl maltoside, Tween 20, Brij 35, or sodium cholate. The soluble extract (4.5 ml) was mixed with 150 μl of α mBax 5B7 or α uBax 6A7 antibody beads either in the absence or in the presence of appropriate detergents 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 0.2% of the appropriate detergent. The bound proteins were then eluted off the beads with 180 μl of 0.1 M acetic acid containing 0.2% Triton X-100. The acid eluants were neutralized with 30 μl of 1 M Tris, pH 8.0.

For the study of Bax homodimerization, murine and rat thymocytes were hypotonically lysed at a cell density of 1×10^8/ml. The soluble proteins from the two cell types were prepared as described above. The soluble extracts were mixed 1:1 and used for the immunoprecipitation studies using the α mBax 5B7 antibody Sepharose beads in the presence of various detergents as described above.

Alternatively, immunoprecipitation of Bax was carried out from detergent-solubilized whole cell lysate. Murine thymocytes were solubilized in 10 ml of 10 mM Hepes, pH 7.4, 150 mM NaCl, and in the presence of 1% Triton X-100, Triton X-114, polydocanol, Chaps, octyl glucoside, or dodecyl maltoside or 0.5% W-1 at a cell density of 5×10^7/ml. The lysate was spun at 14,000 rpm in a Sorvall SA 600 rotor for 15 min to pellet the nuclei and unsolubilized material. Bax complex was then immunoprecipitated from 4.5 ml of the detergent-solubilized lysate with 150 μl of the α mBax 5B7 antibody beads as described above.

For the study of Bax heterodimerization in dexamethasone-treated thymocytes, murine thymocytes (7.5×10^7/ml) in Iscove's medium were subjected to treatment with 2 μM dexamethasone for 4 h. The cells were then collected, spun down, and solubilized in 10 mM Hepes, pH 7.4, 150 mM NaCl containing 1% Triton X-100 or Chaps. Immunoprecipitation of Bax was carried out as described above. For the above immunoprecipitation studies, all lytic, solubilization, and washing buffers contain proteolytic inhibitors (25 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) as described previously (24).

Purification of Bax—Soluble protein extracts from murine thymocytes were prepared as described above except that the lysate was prepared at a cell density of 1×10^8/ml. One hundred and eighty ml of the extract in the presence of 38 mM NaCl was loaded onto a 20-ml AF-heparin-650M column. Flow-through from the column was collected and then loaded onto a 15-ml Fractogel EMD TMAE-650M anion exchange column equilibrated in the same buffer. After washing the column with 3 column volumes of the lysis buffer, Bax was eluted off the column with 3 column volumes of the elution buffer (10 mM Hepes, pH 7.4, and 125 mM NaCl). The eluant was then incubated with 0.5 ml of α mBax 5B7 antibody beads for 3 h at 4 °C. The beads were then washed, and the bound Bax was eluted off the beads with 0.1 M acetic acid containing 0.1% Triton X-100. Six fractions of 0.3-ml samples were collected and neutralized with 40 μl of 1 M Tris, pH 8.0.

Gel Filtration Molecular Weight Sizing of Bax—-The relative molecular weight of the cytosolic Bax was determined by gel filtration over a Sephacryl S-100 column. Murine thymic soluble extract (1×10^8/ml) was loaded onto a TMAE 650M anion exchange column as described above to concentrate the Bax. Half a ml of the 0.125 M NaCl eluant was then loaded onto the gel filtration column (74 ml) equilibrated in 10 mM Hepes, pH 7.4, and 150 mM NaCl. One-ml fractions were collected and analyzed by Western blotting with α uBax 2C8 antibody. The peak fraction containing murine Bax was assigned as the elution volume (V_e) for the purpose of calculating its molecular weight. Blue dextran (2×10^3 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) were run separately as molecular weight standards.

Cross-linking of Bax—For whole cell cross-linking of dexamethasone-treated thymocytes using membrane-permeable cross-linkers, thymocytes were treated with 2 μM dexamethasone for 4 h (23). The cells were washed once in PBS and resuspended in PBS at a cell density of 2.5×10^7/ml. Ten ml of the cell suspension were then incubated with 1 mM DSP or DSG for 30 min on ice. The reaction was quenched with 10 mM Tris, pH 7.4, and 100 mM glycine buffer. The cells were then spun down, and the cell pellet was resuspended in 2 ml of 1% SDS. The samples were run on a 10% SDS-polyacrylamide gel in the absence of β-mercaptoethanol and analyzed by Western blotting.

Binding of Iodinated Antibodies to Thymocyte Membranes—Ten micrograms of anti-murine Bax 5B7, anti-human Bax 1F6, and anti-universal Bax 6A7 monoclonal antibodies were iodinated with Bolton-Hunter reagent according to the manufacturer's protocol. Murine thymocytes were treated with dexamethasone for 4 h and subjected to hypotonic lysis and Dounce homogenization at a cell density of 5×10^7/ml as described above. The lysate (0.5 ml) was adjusted to 150 mM NaCl and incubated with 50 ng (2 μCi/μg) of iodinated antibodies either with or without the presence of 50 μg of cold antibodies for 30 min on ice. The samples were then subjected to ultracentrifugation at 61,000 rpm for 30 min in a TLA 120.1 rotor. The radioactivity of the membrane pellets was then determined with a gamma counter.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gel unless specified) and Western blotting were carried out as described previously (24). For immunoblotting analysis, the blots were probed with either 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), α rBax 1D1 (1:10 diluted culture fluid), or α uBax 2C8 (1:20 diluted culture fluid) diluted in the blocking buffer for 45 min. The blots were then washed in PBS, 0.05% Tween 20 and incubated in blocking buffer containing 1:7000 diluted sheep anti-mouse immunoglobulin peroxidase for an additional 30 min. The blots were again washed in PBS, 0.05% Tween 20 and then in PBS and visualized by ECL Western blotting detection kit.

RESULTS

Epitope Mapping of Anti-Bax Antibodies—The N-terminal epitopes of several anti-Bax monoclonal antibodies were determined by peptide mapping (SPOTs) analysis of a nested set of peptides corresponding to the N-terminal segments of Bax used for the generation of antibodies. As shown in Fig. 1, the binding specificity of α mBax 5B7, α hBax 1F6, and α rBax 1D1 monoclonal antibodies lies within amino acids 7–14. The first 4

[1] The abbreviations used are: DSG, disuccinimidyl glutarate; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; DSP, dithiobis(succinimidyl propionate).

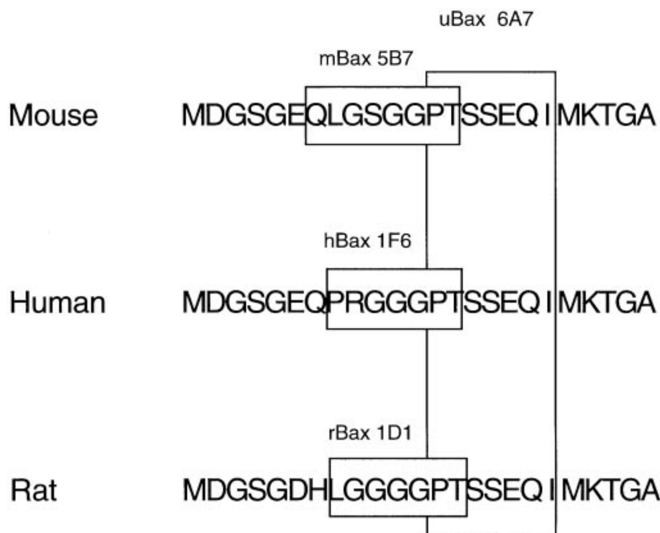

FIG. 1. **Epitope mapping of anti-Bax antibodies.** The epitopes for the various anti-Bax monoclonal antibodies α mBax 5B7, α hBax 1F6, α rBax 1D1, and α uBax 6A7 were mapped by SPOTs analysis. The amino acid residues within the antibody binding sites are *bracketed*.

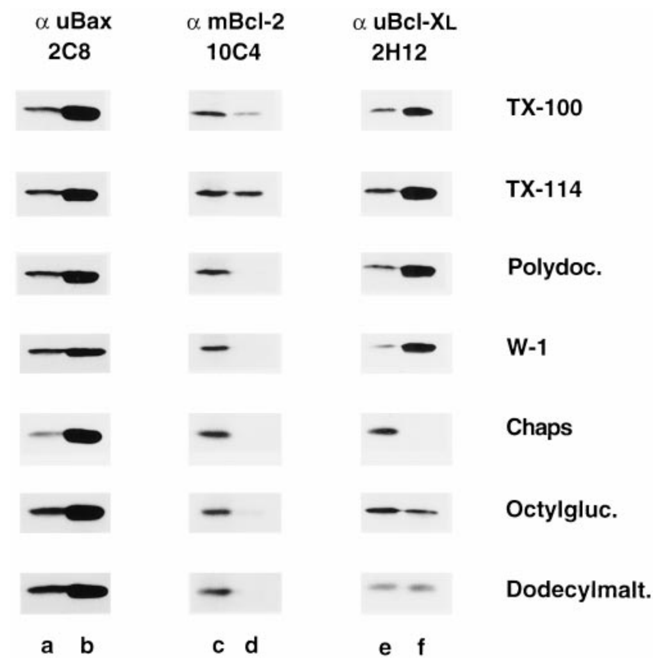

FIG. 2. **Detergent-dependent heterodimerization of Bax with Bcl-2 and Bcl-X_L.** Murine thymocytes were solubilized in the presence of Triton X-100, Triton X-114, polydocanol, W-1, Chaps, octyl glucoside, and dodecyl maltoside. Bax was immunoprecipitated from the detergent-solubilized thymocyte lysates by α mBax 5B7 antibody Sepharose beads. The presence of Bax (*lane b*), Bcl-2 (*lane d*), and Bcl-X_L (*lane f*) in the immunoprecipitated samples was detected by Western blotting analyses with α mBax 5B7 (*left*), α mBcl-2 10C4 (*middle*), and α uBcl-X_L 2H12 (*right*) monoclonal antibodies. *Lanes a, c*, and *e* represent endogenous Bax, Bcl-2, and Bcl-X_L in murine thymocyte lysates, respectively.

amino acids within this particular region are distinct between mouse, human, and rat and appear to account for the species specificity of these antibodies. The epitope for the α uBax 6A7 monoclonal antibody, produced against a peptide sequence (amino acids 12–24) common to murine, human, and rat Bax, was found within amino acids 13–19 which partially overlaps with epitopes of the above-described species-specific antibodies.

Detergent-dependent Selective Heterodimerization of Bax with Bcl-2 and Bcl-X_L—The propensity of Bax to form Bax/Bcl-2 and Bax/Bcl-X_L heterodimers in the presence of nonionic detergents Triton X-100 and Nonidet P-40 (24) led us to investigate the effect of other detergents in the induction of this process. Murine thymocytes were solubilized in either Triton X-100, Triton X-114, polydocanol, W-1, Chaps, octyl glucoside, or dodecyl maltoside. Immunoprecipitation of Bax was then carried out in the presence of these detergents using α mBax 5B7 monoclonal antibody (Fig. 2). Detergents such as Tween 20, Brij 35, or sodium cholate failed to efficiently solubilize the thymocytes, and therefore they were excluded from this experiment. The immunoprecipitated samples were analyzed by Western blotting with α uBax 2C8, α mBcl-2 10C4, and α uBcl-X_L 2H12 monoclonal antibodies for the detection of murine Bax, Bcl-2, and Bcl-X_L, respectively. As shown in Fig. 2, Triton X-100 and its related homolog Triton X-114 readily enable Bax/Bcl-2 and Bax/Bcl-X_L heterodimer formation. Other detergents such as octyl glucoside mediate Bax/Bcl-X_L heterodimer formation but allow much less Bax/Bcl-2 heterodimer. Meanwhile, detergents such as polydocanol, W-1, and dodecyl maltoside enable only Bax/Bcl-X_L formation with a clear absence of Bax/Bcl-2 heterodimer. Finally the zwitterionic detergent Chaps allows neither Bax/Bcl-2 nor Bax/Bcl-X_L heterodimer formation. Addition of 0.1% SDS to the Triton X-100 solubilization buffer disrupts Bax/Bcl-2 heterodimer formation but does not affect Bax heterodimerization to Bcl-X_L (data not shown), suggesting that the interaction between Bax and Bcl-X_L is comparably stronger than that of Bax and Bcl-2. Based on this study, it appears that different detergents may induce different sets of conformational changes in Bax, and perhaps in Bcl-2 and Bcl-X_L as well, to facilitate the differential heterodimerization of Bax with Bcl-2 or Bcl-X_L.

Effect of Detergent on the Homodimerization of Bax—We have previously reported that Bax homodimerization, like heterodimerization, appears to be a detergent-dependent process (24). Since we find Bax heterodimerization to be dependent upon the type of detergent present, we set out to examine the effect of different detergents on Bax homodimerization. Bax was immunoprecipitated from a mixture of the soluble protein extracts of murine and rat thymocytes either in the absence of detergent or in the presence of Triton X-100, Triton X-114, polydocanol, W-1, Chaps, octyl glucoside, dodecyl maltoside, Tween 20, Brij 35, or sodium cholate. Murine Bax was immunoprecipitated from the mixture by α mBax 5B7 monoclonal antibody to examine its propensity to dimerize with rat Bax. The immunoprecipitated samples were analyzed by Western blotting with anti-murine Bax 5B7 and anti-rat Bax 1D1 monoclonal antibodies (Fig. 3, *top* and *middle*). As revealed by Western blotting, α mBax 5B7 antibody immunoprecipitated murine Bax under all conditions. Homodimerization of murine Bax to rat Bax, as determined by Western blotting with anti-rat Bax 1D1 antibody, occurs most readily in Triton X-100 and Triton X-114 (Fig. 3, *middle, lanes e* and *f*) and is reduced in the presence of polydocanol and dodecyl maltoside (Fig. 3, *middle, lanes g* and *k*). Bax homodimers do not form in the absence of detergent (Fig. 3, *middle, lane d*), as expected, nor in the presence of W-1, Chaps, octyl glucoside, Tween 20, Brij 35, and sodium cholate (Fig. 3, *middle, lanes h, i, j, l, m*, and *n*). To determine if Bax heterodimerization with Bcl-X_L occurs under similar conditions, the above-described immunoprecipitated samples were analyzed by Western blotting with α uBcl-X_L 2H12 antibody. The results indicate that Bax can differentially form heterodimers with Bcl-X_L in the presence of most of these detergents with the exception of Chaps and Brij 35 (Fig. 3, *bottom, lanes i* and *m*). In the absence of detergent, as previously reported (24), Bax does not heterodimerize with Bcl-X_L (Fig. 3, *bottom, lane d*). These results suggest that the Bax homodimerization state is not only dependent upon the type of detergent but also appears to be distinct from its heterodimer-

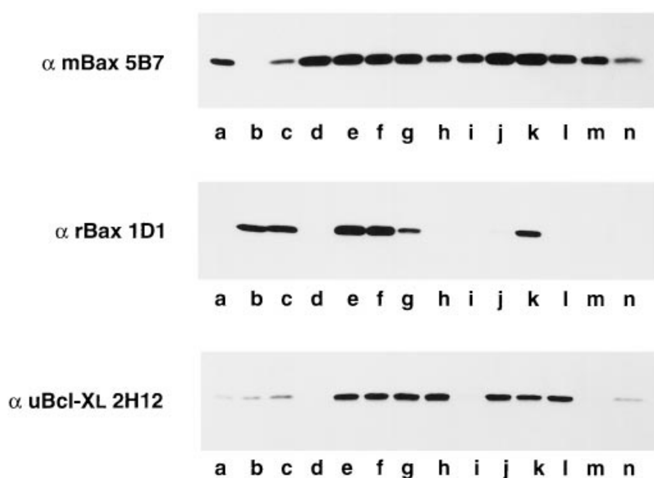

FIG. 3. **Distinct states of Bax homo- and heterodimerization induced by detergents.** Immunoprecipitation of Bax from a mixture of the soluble protein extracts of murine and rat thymocytes was carried out using α mBax 5B7 antibody Sepharose beads either without (*lane d*) or in the presence of Triton X-100 (*lane e*), Triton X-114 (*lane f*), polydocanol (*lane g*), W-1 (*lane h*), Chaps (*lane i*), octyl glucoside (*lane j*), dodecyl maltoside (*lane k*), Tween 20 (*lane l*), Brij 35 (*lane m*), and sodium cholate (*lane n*). The protein samples were analyzed by Western blotting with either α mBax 5B7 (*upper panel*), α rBax 1D1 (*middle panel*), or α uBcl-X_L 2H12 (*lower panel*) monoclonal antibodies. *Lane a*, soluble protein extract of murine thymocytes; *lane b*, soluble protein extract of rat thymocytes; and *lane c*, combined soluble protein extracts of murine and rat thymocytes.

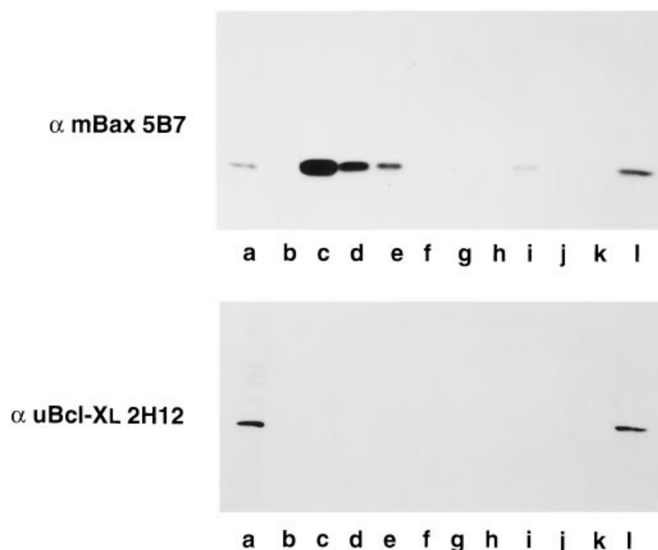

FIG. 4. **Detergent-dependent binding of the conformation-sensitive anti-uBax 6A7 monoclonal antibody to Bax.** Anti-uBax 6A7 monoclonal antibody was used to immunoprecipitate Bax from the soluble protein extract of murine thymocytes (*lane a*) either in the absence of detergent (*lane b*) or in the presence of Triton X-100 (*lane c*), Triton X-114 (*lane d*), polydocanol (*lane e*), W-1 (*lane f*), Chaps (*lane g*), octyl glucoside (*lane h*), dodecyl maltoside (*lane i*), Tween 20 (*lane j*), Brij 35 (*lane k*), or sodium cholate (*lane l*). Western blotting analyses of the immunoprecipitated samples were carried out with α mBax 5B7 and α uBcl-X_L 2H12 monoclonal antibodies.

ization state.

Detergent-dependent Exposure of the α uBax 6A7 Antibody Epitope—We have previously described the exposure of an N-terminal epitope of Bax (amino acids 12–24), which contains the binding site for the α uBax 6A7 antibody, in the presence of Triton X-100 and Nonidet P-40 (24). In order to further investigate the conformation-dependent exposure of this epitope that correlates with Bax hetero- and homodimerization and competes for Bax-Bcl-X_L heterodimer formation, we extended the immunoprecipitation study with α uBax 6A7 antibody in the presence of various detergents. The immunoprecipitated samples were analyzed by Western blotting with α mBax 5B7 monoclonal antibody for the detection of murine Bax bound to the 6A7 antibody. Whereas the α uBax 6A7 antibody binds strongly to Bax in the presence of Triton X-100 (Fig. 4, *top, lane c*), a decreased affinity was observed in the presence of Triton X-114, polydocanol, and sodium cholate (Fig. 4, *top, lanes d, e* and *l*), and only trace binding was observed in the presence of dodecyl maltoside (Fig. 4, *top, lane i*). Bax did not bind to the uBax 6A7 antibody either in the absence of detergent or in the presence of W-1, Chaps, octyl glucoside, Tween 20, and Brij 35 (Fig. 4, *top, lanes b, f, g, h, j*, and *k*). Thus, the epitope for the 6A7 antibody (now mapped to amino acids 13–19), which is normally buried, apparently adapts the exposed conformation only in the presence of selected detergents.

We have also previously reported that the 6A7 antibody binding site competes with heterodimer formation (24). Here we examined the effect of different detergents on this competitive process by analyzing the 6A7 antibody immunoprecipitated Bax samples for possible heterodimer formation with Bcl-X_L. As shown in the Western blot probed with α uBcl-X_L 2H12 antibody, Bax immunoprecipitated in the presence of Triton X-100, Triton X-114, polydocanol, and dodecyl maltoside is not associated with cytosolic Bcl-X_L (Fig. 4, *bottom Western, lanes c–e* and *i*). Surprisingly, Bax immunoprecipitated by the 6A7 antibody in the presence of sodium cholate forms heterodimer with Bcl-X_L (Fig. 4, *bottom Western, lane l*). This suggests that the detergent environment by which Bax is exposed to may greatly dictate the conformation undertaken by this protein.

Purification and Molecular Weight Sizing of Bax—Our recent finding that Bax is a soluble protein prompted us to determine if Bax in its cytosolic state is associated with any binding proteins. Bax was purified from the murine thymocyte extract by sequential chromatography through heparin, TMAE anion exchange, and anti-mouse Bax 5B7 antibody columns. The initial step with heparin column removed a significant quantity of cytosolic proteins without retaining Bax (Fig. 5, *lanes b*). The subsequent step by TMAE anion exchange chromatography retained the majority of proteins while allowing Bax to elute at 0.125 M NaCl (Fig. 5, *lane d*). Incidentally, Bcl-X_L elutes at a salt concentration of between 0.3 and 0.5 M, further suggesting that the cytosolic Bax is not associated with Bcl-X_L. Bax was purified from the TMAE column eluant by anti-mouse Bax 5B7 antibody Sepharose beads. As shown in Fig. 5 (*lane e*), the purified Bax displays an apparent molecular mass of 22 kDa, and it does not appear to be associated with any other proteins.

To confirm that the cytosolic Bax is a monomeric and not a dimeric or multimeric protein, gel filtration chromatography was carried out on a Sephacryl S-100 column using blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A as molecular weight standards. As shown in Fig. 6, the apparent molecular mass of the murine thymic cytosolic Bax was calculated to be 25 kDa. This suggests that murine Bax in healthy thymocytes is a monomeric protein and does not appear to be bound with any other proteins.

Effect of Apoptosis on Bax Dimer Formation and Exposure of the 6A7 Epitope—Induction of apoptosis in murine thymocytes results in the insertion of a significant fraction of Bax from the cytosol into membranes (23, 38). Thus it remained plausible that Bax inserts into membranes and adopts the detergent-induced conformation to dimerize with Bcl-2 and Bcl-X_L. In order to determine if this insertion process resulted in the

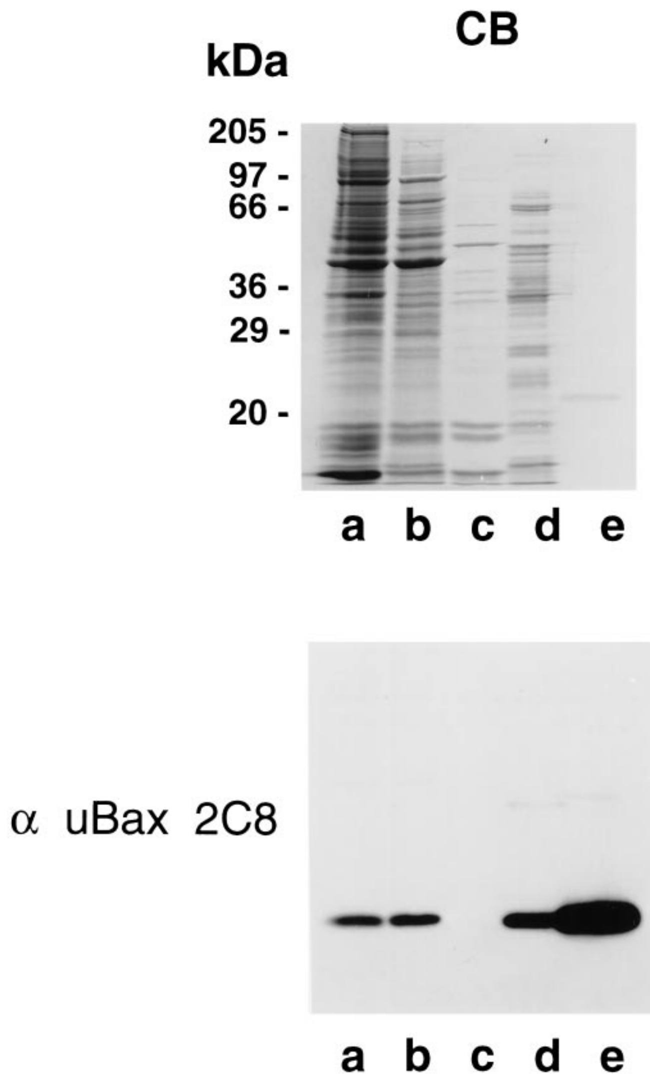

FIG. 5. **Purification of Bax from murine thymocytes.** Murine Bax was purified from thymocyte soluble protein fraction by heparin affinity, TMAE anion exchange, and α mBax 5B7 immunoaffinity chromatography. The protein samples were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue or by Western blotting analysis with α uBax 2C8 monoclonal antibody. *Lane a*, soluble murine thymocyte extract; *lane b*, flow-through from heparin column; *lane c*, flow-through from TMAE anion exchange column; *lane d*, eluant from TMAE column; *lane e*, eluant from α mBax 5B7 antibody Sepharose beads.

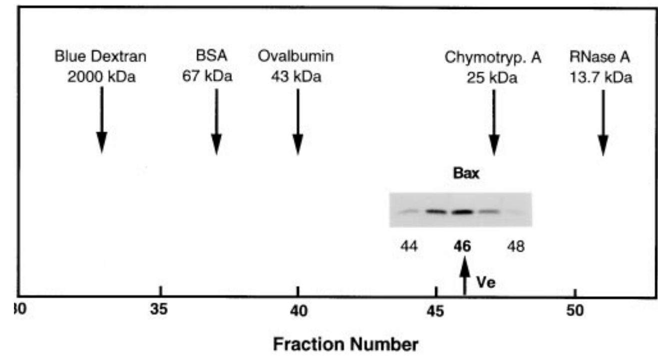

FIG. 6. **Gel filtration molecular weight sizing of murine Bax.** The relative molecular weight of murine thymocyte Bax was determined by gel filtration over a Sephacryl S-100 column. Blue dextran, bovine serum albumin, chymotrypsinogen A, and ribonuclease A were used as standards. The V_e of Bax, as determined by Western blotting, is 46 ml.

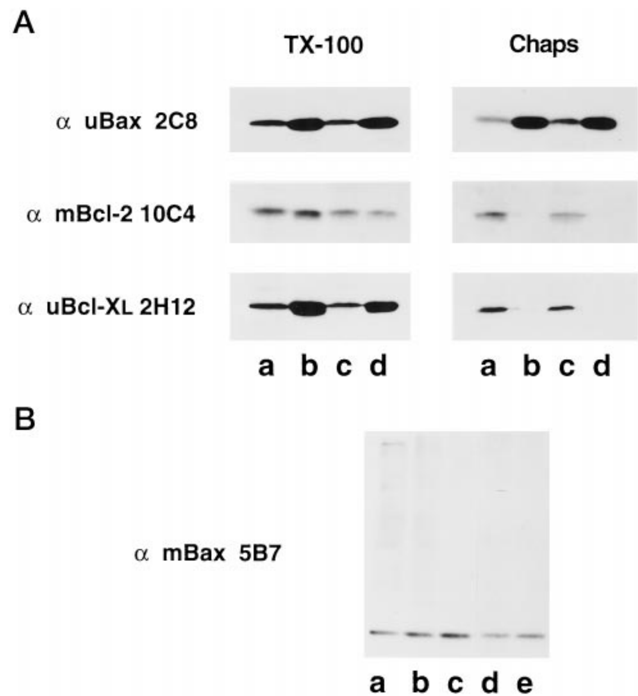

FIG. 7. **Quaternary state of Bax during apoptosis.** *A*, immunoprecipitation of Bax was carried out with α mBax 5B7 antibody from either healthy murine thymocytes (*lanes a* and *b*) or thymocytes treated with dexamethasone (*lanes c* and *d*). The study was carried out in the presence of Triton X-100 (*left column*) or Chaps (*right column*). The protein samples were probed by Western blotting with α uBax 2C8, α mBcl-2 10C4, and α uBcl-X_L 2H12 monoclonal antibodies. *Lanes a* and *c* are detergent-solubilized thymocyte lysates, and *lanes b* and *d* are the immunoprecipitated samples. *B*, healthy thymocytes (*lanes b* and *c*) and thymocytes treated with dexamethasone (*lanes d* and *e*) were analyzed by chemical cross-linking with DSG (*lanes b* and *d*) or DSP (*lanes c* and *e*). The cross-linked protein samples were analyzed by Western blotting with α mBax 5B7 monoclonal antibody. *Lane a* is the uncross-linked thymocyte sample.

formation of Bax heterodimers with Bcl-2 or Bcl-X_L, we performed immunoprecipitation of Bax from dexamethasone-treated thymocytes in the presence of either Triton X-100 or Chaps. As shown in Fig. 7*A*, the relative amount of Bax/Bcl-2 and Bax/Bcl-X_L heterodimers obtained from Bax immunoprecipitation in the presence of Triton X-100 does not significantly change before or after the induction of apoptosis (Fig. 7*A, left*). However, since Triton X-100 induces Bax dimer formation, it is not possible to differentiate dimer formation resulting from the detergent or the apoptosis.

Since Chaps can solubilize thymocytes without triggering Bax dimer formation, we carried out immunoprecipitation of Bax from apoptotic thymocytes using this detergent. We have first determined that the presence of Chaps does not disrupt Bax heterodimer formation when used in combination with Triton X-100 (data not shown). However, either before or after induction of apoptosis (Fig. 7*A, right*), no Bax heterodimers were found in Chaps-solubilized thymocyte lysate, suggesting that Bax heterodimer formation does not occur during apoptosis. We also investigated the exposure of the 6A7 antibody epitope in the membrane-bound Bax. This was carried out by mixing the apoptotic thymocyte lysate with various iodinated antibodies either in the presence or absence of competing cold antibodies. As shown in Table I, a significant binding of the 6A7 antibody was observed in apoptotic membranes of murine thymocytes, suggesting that this epitope may be exposed once Bax inserts into membranes.

We further examined the possibility of Bax dimer formation in membranes during apoptosis by cross-linking of whole thy-

mocytes with DSG and DSP. Although the Coomassie Blue-stained gel showed extensive cross-linking (data not shown), we failed to observe the formation of Bax dimers (Fig. 7*B*). Similarly we did not detect any Bax dimer formation in a cross-linking study of organelle membranes in HL-60 cells treated with staurosporine (data not shown). Nevertheless, it is possible that the lysine residues that are primary sites for cross-linking are not exposed in Bax. These studies suggest that the formation of Bax dimer may not occur physiologically and that dimer formation induced by nonionic detergents such as Triton X-100 and Nonidet P-40 does not truly represent the state of Bax within cells.

DISCUSSION

The pro-apoptotic factor, Bax, has been described as an integral membrane protein that regulates apoptosis through the formation of homo- or heterodimers (1, 32). However, we have shown here and in a previous report (24) that in healthy murine thymocytes, Bax is a soluble protein and is monomeric based on subcellular fractionation, immunoaffinity purification, and gel filtration molecular weight sizing analyses. In addition we have shown that the propensity of Bax to form dimers lies with its conformational change induced by nonionic detergents. In this report we have further examined Bax conformational change induced by a variety of detergents with respect to its dimer formation and the exposure of a conformation-sensitive epitope for the 6A7 antibody (24). As shown in Table II, both the dimerization of Bax and the exposure of its 6A7 antibody binding site appear to be highly dependent upon the type of detergent.

Based on studies by site-directed mutagenesis, immunoprecipitation and yeast two-hybrid select system, dimer formation among members of the Bcl-2 family has been shown to be mediated through BH1 and BH2 domains in pro-survival factors such as Bcl-2 and Bcl-X_L (39) and BH3 domains in pro-apoptotic factors such as Bax, Bak, Bid, Bik, Bad, and Hrk (7, 11, 14, 40–43). However, the physiological role of these dimer formations is still unclear. Whereas the majority of studies suggest that these dimerization domains are essential for both the dimer formation and the anti- or pro-apoptotic activities of these proteins, a few studies have suggested that dimer formation may be unrelated to the apoptosis regulatory activities of these proteins (33–35). All these binding studies reported so far, however, were carried out in the presence of Nonidet P-40. Our earlier finding of Bax being a soluble protein (24) circumvented the need for the addition of detergents. Our studies suggest that the dimeric state of Bax correlates with a nonionic detergent-induced conformational change that perhaps leads to the exposure of the BH3 domain of this protein and perhaps the BH1 and BH2 domains of Bcl-2 and Bcl-X_L as well. Thus the relative ratio of Bax homodimers to Bax heterodimers obtained by previous studies does not truly reflect the actual dimeric states of these proteins in cells but rather ones that are favored by the specific conformation called for by the detergent chosen for the experiment.

We have analyzed Bax dimerization and conformational changes with the following three classes of detergents: nonionic with polyoxyethylene (Nonidet P-40, Triton X-100, Triton X-114, polydocanol, W-1, Tween 20, and Brij 35) or sugar head groups (octyl glucoside and dodecyl maltoside), zwitterionic (Chaps), and anionic (sodium cholate). Within the polyoxyethylene subclass of non-ionic detergents, detergents with alkylphenyl hydrophobic moieties (Nonidet P-40, Triton X-100, and Triton X-114) more readily induce Bax dimer formation and the exposure of the 6A7 antibody epitope as compared with those with alkyl (polydocanol, W-1, and Brij 35) or acyl sorbitan ester (Tween 20) groups. The length of the polyoxyethylene chains in detergents with alkyl head groups also appears to play a determinant role. Whereas polydocanol ($n = 9$) and W-1 (a mix of 64 parts $n = 10$ and 36 parts $n = 20$) gave at least some extent of Bax dimerization and 6A7 epitope exposure, Brij 35 ($n = 23$), despite having the same alkyl hydrophobic group as polydocanol, fails to elicit either response. Alkyl glucosides (octyl glucoside and dodecyl maltoside) gave varying degrees of Bax dimers but with little or no 6A7 antibody epitope exposure.

The zwitterionic detergent Chaps that has both the bile salt hydrophobic and sulfobetaine polar groups is the only detergent we tested that can solubilize thymocytes but does not promote Bax dimer formation or 6A7 antibody epitope exposure. In contrast, the bile salt detergent sodium cholate, from

Table I
Epitope exposure of Bax in membranes after apoptosis

Monoclonal antibody	CPM bound ± S.D.	Specific cpm
5B7	27,373 ± 4,604	15,612
5B7 plus cold 5B7	11,761 ± 2,026	
6A7	22,306	5,825
6A7 plus cold 1F6	23,108 ± 2,995	
6A7 plus cold 6A7	16,481 ± 4,586	
1F6	20,568 ± 629	2,227
1F6 plus cold 1F6	18,341 ± 411	

Table II
Bax dimer formation and the exposure of the 6A7 antibody epitope in the presence of different detergents
The relative extent of Bax dimerization and 6A7 antibody binding were assigned as strong (+++), medium (++), weak (+), or none (−).

	Heterodimerization			Homodimerization	6A7 binding
	Bcl-2[a]	Bcl-X_L[a]	Bcl-X_L[b]		
None	N/A[c]	N/A	−	−	−
Nonidet P-40[d]	++	+++	+++	+++	+++
Triton X-100	++	+++	+++	+++	+++
Triton X-114	+++	+++	+++	+++	++
Polydocanol	−	+++	+++	++	++
W-1	−	+++	+++	−	−
Chaps	−	−	−	−	−
Octyl glucoside	+	++	+++	−	−
Dodecyl maltoside	−	++	+++	++	+
Tween 20	N/A	N/A	+++	−	−
Brij 35	N/A	N/A	−	−	−
Sodium cholate	N/A	N/A	+	−	++

[a] Results obtained from detergent-solubilized thymocytes.
[b] Results obtained from soluble protein extract of thymocytes.
[c] N/A, not applicable.
[d] Results taken from Hsu and Youle (24).

which Chaps was derived, allows both the heterodimer formation of Bax to Bcl-X_L and the exposure of the 6A7 epitope. However, Bax immunoprecipitated by the 6A7 antibody in the presence of sodium cholate readily forms heterodimers with Bcl-X_L, whereas Bax immunoprecipitated by this antibody in the presence of alkylphenyl-based detergents is not associated with either Bcl-2 or Bcl-X_L due to possible steric hinderance (24). Thus the conformational change leading to the exposure of the 6A7 epitope by sodium cholate appears to be different from those induced by Triton X-100, Triton X-114, and Nonidet P-40.

We have uncovered four major conformational states of Bax. In the first state that pertains to the absence of detergent or the presence of Chaps, Bax undertakes a conformation in which it neither dimerizes nor exposes the 6A7 epitope. In the second state represented by exposure to W-1 and Tween 20, Bax can undergo heterodimerization but not homodimerization nor exposure of the 6A7 epitope. The third major state can be typified by the sodium cholate-induced heterodimerization of Bax and the exposure of the 6A7 epitope. The fourth state is best represented by the one induced by Triton X-100, Triton X-114, and Nonidet P-40 in which Bax can undergo both homo- and heterodimerization and exposure of the 6A7 epitope. However, unlike the third state induced by sodium cholate, the heterodimerization process induced by alkylphenyl detergents competes with the binding of the 6A7 antibody. So far we have not detected dimer formation in apoptotic thymocytes by chemical cross-linking or immunoprecipitation in the presence of Chaps. However, we have detected some binding of the 6A7 antibody to the apoptotic membranes. Thus, questions remain as to the actual state of Bax conformation during apoptosis.

We have recently described the redistribution of Bax from the cytosol into membranes during apoptosis (23, 38) and the importance of the hydrophobic tail of Bax in promoting this insertion process (38). Interestingly, the recent x-ray crystallographic analysis of Bcl-X_L indicates that this protein folds much like the translocation domain of diphtheria toxin (44), which is capable of forming pores for the translocation of its toxin domain (45). Subsequently, it was shown that Bcl-2, Bcl-X_L, and Bax can form channels to allow ion conductivity (46–49). However, the recombinant protein samples from these studies lack the C-terminal hydrophobic tail, and during their preparation they were exposed to Triton X-100, a detergent we described in our studies as having the ability to alter the conformation of Bax. In our studies we have found a conformation-dependent exposure of the 6A7 antibody epitope on Bax in apoptotic membranes but with no dimer formation. This membrane-associated conformation appears to be intermediary of the ones found in Chaps in which Bax neither dimerizes nor exposes its 6A7 epitope and in alkylphenyl detergents in which Bax dimerizes and exposes its 6A7 epitope. Thus interesting challenges remain in defining not only the molecular basis and consequence of Bax insertion but also the conformational change associated with the insertion.

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