Title: Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis

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Running title: Lipid-induced changes in Bax conformation
SUMMARY

The Bcl-2 family member Bax is an apoptosis-promoting protein that normally resides in an inactive state within the cytoplasm of healthy cells. Upon induction of apoptosis by diverse stimuli, Bax undergoes a conformational change and translocates to mitochondria, where it oligomerizes and forms pores that allow the release of cytochrome c and other cytotoxic factors. Protein-protein interactions between Bax and other Bcl-2 family members are strongly implicated in Bax activation, but a compelling case has recently been made for the involvement of lipids in this process as well. Here we report that purified Bax undergoes a reversible conformational change upon incubation with lipid vesicles in the absence of other proteins. This Bax-liposome interaction does not depend on a specific lipid composition. Changes in Bax conformation were observed by immunoprecipitation with the conformation-specific antibody 6A7, circular dichroism spectroscopy, and differential scanning calorimetry. Although liposomes induced Bax to become 6A7-reactive (a feature normally associated with the onset of apoptosis), the protein did not insert into membranes, become oligomeric, or form pores, clearly indicating that other triggers are required for Bax to achieve its final pro-apoptotic state. Indeed, the lipid-induced Bax conformational change is shown to be required for tBid-induced Bax oligomerization and pore formation, putting it upstream of tBid activity in this molecular pathway to Bax activation. These data demonstrate that Bax is sensitized to activation by transient interaction with lipid membrane surfaces, and provide evidence that Bax activation proceeds in a stepwise fashion, with multiple triggers and potential levels of regulation.
INTRODUCTION

Programmed cell death in multicellular organisms is regulated by the Bcl-2 family of proteins (1). Some proteins within this family promote apoptosis while others promote survival, but the exact molecular mechanisms by which these proteins function are still largely unknown. Bax is a pro-apoptotic member of the Bcl-2 family of cell death regulators (2). In healthy cells, Bax resides in the cytoplasm (3), but upon induction of apoptosis by diverse stimuli, Bax undergoes a conformational change involving both its N- and C-termini (4,5), and translocates to and inserts into membranes of the mitochondria and endoplasmic reticulum (ER) (6,7). In the mitochondrial outer membrane, Bax becomes homooligomeric (8) and forms pores (9) that allow the release of cytochrome c and other pro-apoptotic factors. Bax at the ER is implicated in the regulation of calcium ion fluxes (10,11), which can lead to opening of the permeability transition pore in mitochondria, again resulting in cytochrome c release and caspase activation.

Regulation of Bax activity is thus a critical determinant of cell fate. Protein-protein interactions between Bax and other Bcl-2 family members are key to this regulation. For example, Bid is a pro-apoptotic member of the Bcl-2 protein family that is processed by proteolytic cleavage to an activated form (tBid) in response to death receptor signaling (12), and tBid subsequently functions as a potent Bax activator to induce apoptosis (13,14). Conversely, Bcl-X_L is an anti-apoptotic Bcl-2 family protein that has been reported to heterodimerize with Bax and nullify its activity (15). Regulation of Bax activity may also depend on interactions with non-Bcl-2 family members; Ku70 (part of the regulatory subunit of DNA-dependent protein kinase) and humanin (a 24 amino acid anti-apoptotic peptide encoded in mammalian genomes) have both been shown to interact with Bax in the cytoplasm and prevent its translocation to membranes (16,17).
A compelling case has also been made that lipids play a role in Bax pore formation. For example, in the absence of other proteins, the permeabilization of liposomes induced by detergent-activated Bax has been shown to depend on both intrinsic monolayer curvature (18) and on the mitochondria-specific lipid cardiolipin (19,20). In addition, visualization of apoptotic cells by confocal microscopy shows that Bax oligomerization occurs primarily at discrete foci on mitochondria, which subsequently become sites of mitochondrial fission (21). Based on these and other data, it has been hypothesized that Bax may function to disrupt membranes via formation of non-specific lipidic pores, such as those observed during membrane fission and fusion events (reviewed in 22,23). Such lipid-lined pores would be large enough to allow the release of not only cytochrome c from the mitochondrial intermembrane space, but also the release of other much larger apoptotic inducers such as Smac/Diablo (27 kDa), EndoG (33 kDa), and AIF (67 kDa). Indeed, tBid-activated Bax is reported to be capable of permeabilizing liposomes to dextrans of up to 2000 kDa (20). But apart from their complicity in actual pore formation, do lipids play an additional role in Bax activation?

To answer this question and further characterize the molecular mechanism of Bax activation, we examined whether purified recombinant Bax could interact with liposomes of mitochondrial membrane-like composition by testing for all the in vitro hallmarks of Bax activation: conformational changes, membrane binding and insertion, oligomerization, and pore formation. Our data demonstrate that Bax undergoes a transient conformational change upon interaction with a lipid membrane surface, indicating a previously unrecognized role for lipids in the regulation of Bax activity. Further, this Bax-lipid interaction is shown to be a prerequisite for tBid-induced Bax oligomerization and pore formation, highlighting the biological significance of this conformational change to Bax activation.
EXPERIMENTAL PROCEDURES

Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purified from egg. Phosphatidylinositol (PI) was from bovine liver. Phosphatidylserine (PS) and cardiolipin were purchased as synthetic lipids in dioleoyl (DO) or tetraoleoyl form, respectively. 8-Aminonaphthalene 1,3,6-trisulfonic acid (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were from Molecular Probes (Eugene, OR). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (HEPES) were from BioShop Canada Inc. (Burlington, ON, Canada). Other chemicals were purchased from Sigma. ER membranes were purified from canine pancreas (24), and mitochondria were isolated from rat liver (25). Bax monoclonal antibodies 6A7 and 2D2 were generous gifts from Richard Youle.

Protein purification

Recombinant full-length human Bax with no additional amino acid residues was expressed in Escherichia coli and purified as an intein/chitin-binding domain (CBD) fusion as described previously (26). Lysis of E. coli was achieved by mechanical disruption with a French press, and at no point was the protein exposed to detergents that alter the native Bax conformation. After affinity chromatography with a chitin column, intein self-cleavage and release of Bax from its fusion partner was initiated by incubation with buffer containing 100 mM 2-mercaptoethanol for 36 hours. The eluted protein was applied to a column of DEAE Sepharose (Amersham Biosciences, Piscataway, NJ) and collected in the flow-through, with residual impurities remaining bound to the column. Eluted Bax was dialyzed in 10 mM HEPES (pH 7.4), 100 mM NaCl, 0.2 mM EDTA and 30% glycerol, then flash-frozen and stored at -80°C.
Detergent activated Bax was obtained by incubating the purified recombinant protein in buffer containing 1% (w/v) octyl glucoside for 1 hour at 4°C, as previously reported (27).

Recombinant full-length human Bcl-X\textsubscript{L} with no additional amino acid residues was purified as an intein/CBD fusion, as above. CHAPS was added to the lysis and wash buffers to prevent protein aggregation (at 1% and 0.2%, respectively). The protein eluted from the chitin column was further purified by hydrophobic interaction chromatography using a column of Phenyl Sepharose (Amersham Biosciences, Piscataway, NJ). Bcl-X\textsubscript{L} was eluted from the Phenyl Sepharose column in 20 mM Tris, 20% (v/v) glycerol, and 0.2% CHAPS, then flash-frozen and stored at -80°C.

Recombinant full-length rat Bid with an N-terminal 6xHis tag was purified and cleaved with caspase 8 as described previously (28). The C-terminal fragment of cleaved Bid (tBid) was purified away from the N-terminal Bid fragment and residual caspase 8 using nickel-nitrilotriacetic acid agarose (Qiagen) as described (28). Proteins were judged to be greater than 95 percent pure based on Coomassie Blue staining of SDS-PAGE gels (see Fig. 6C).

**Liposome preparation**

Lipids were mixed in the appropriate ratio from stocks dissolved in chloroform. To mimic the composition of the mitochondrial outer membrane, liposomes were made up of PC, PE, PI, DOPS, and tetraoleoylcardiolipin (48:28:10:10:4 molar ratio). The organic solvent was removed by evaporation under a stream of nitrogen gas, followed by incubation for 2 hours in a vacuum to ensure complete solvent removal. Lipid films were resuspended in targeting buffer (10 mM HEPES (pH 7.4), 20 mM KCl, 5 mM MgCl\textsubscript{2}, 0.2 mM EDTA), subjected to 10 freeze-thaw cycles, and large unilamellar vesicles were formed by extrusion through 100 nm Nucleopore polycarbonate membranes. To prepare ANTS/DPX-charged liposomes, 12.5 mM
ANTS and 45 mM DPX were included in the targeting buffer, and non-entrapped fluorophore and quencher were removed by gel filtration using a column of CL2B Sepharose (Amersham Biosciences).

**Immunoprecipitations**

Immunoprecipitations with the conformation-specific antibody 6A7 were performed in 0.5 mL reactions in targeting buffer (see above) at 4°C, unless otherwise specified. Bax and liposomes were added at concentrations of 30 nM and 30 µM, respectively (approximately 300 ng of protein and 12 µg of total lipid). Immunoprecipitation was allowed to occur overnight on a rotator. In the absence of liposomes, Triton X-100 or CHAPS were added at 0.2% (w/v) as positive and negative controls, respectively. Immunoprecipitates were collected by incubating with protein G-Sepharose for 2 hours, followed by centrifugation for 1 min. The pellets were washed two times with wash buffer A (10 mM HEPES (pH 7.4), 150 mM NaCl, 2% CHAPS), three times with wash buffer B (10 mM HEPES (pH 7.4), 150 mM NaCl, 0.2% CHAPS), then an additional three times with wash buffer C (100 mM Tris-HCl (pH 8), 100 mM NaCl). Immunoprecipitates were released from the beads in SDS loading buffer and analyzed by Western blotting with the Bax 2D2 antibody.

To determine the reversibility of Bax 6A7 epitope exposure, protein and liposomes were co-incubated for a period of two hours (in a 0.4 mL volume), and then protein was separated from liposomes over a gel filtration column of CL2B Sepharose with a 4 mL bed volume. The resulting separation time was 30 min (at an approximate flow rate of 0.2 mL/min). The included Bax-containing fractions were pooled and divided into aliquots, and then 6A7 antibody was added at 0, 30, and 60 min post-separation, followed by immunoprecipitation as described above.
Circular dichroism (CD) spectroscopy

CD spectra were recorded using an AVIV Model 215 CD spectrometer from Protein Solutions (Lakeview, NJ, USA). Samples were prepared by diluting a concentrated Bax stock solution into 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA. The concentration of the Bax stock solution was determined by amino acid analysis. Where applicable, lipids (DOPC:DOPE:DOPS:PI:cardiolipin; 43:27:9:9:12 molar ratio) were introduced as a vacuum-dried film that was hydrated in buffer and sonicated to near-clarity before addition of protein. The final concentration of Bax in all CD spectroscopy samples was 2.7 µM. CD spectra were corrected for the buffer baseline, and expressed in terms of mean molar residue ellipticity. Scans were measured twice and averaged. Secondary structure was analyzed with the Selcon3 program (29). Thermal denaturation curves were obtained by recording the temperature dependence of Bax ellipticity at 222 nm. Temperature scans were performed at a heating or cooling rate of 1°C/min. Ellipticity was measured from 25°C to 95°C, and then back to 25°C in order to assess the reversibility of denaturation. To compare the rates of thermal denaturation, the molar ellipticity at 25°C was defined as -1.

Differential scanning calorimetry (DSC)

DSC runs were performed using an N-DSCII microcalorimeter from Calorimetry Sciences Corporation (American Forks, UT, USA). Data analysis was carried out with the program Origin. For sample preparation, concentrated Bax stock solutions were dialyzed against 10 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, and the final dialyzate was used in the buffer reference cell. Sample and buffer were degassed prior to loading. Where applicable, lipids (DOPC:DOPE:DOPS:PI:cardiolipin; 43:27:9:9:12 molar ratio) were introduced as a vacuum-dried film on the walls of a tube (lipid to protein ratio of 1000); the protein solution was added to
the film and mixed by vortexing extensively under argon before degassing and loading. Three independent sets of runs were performed using three independently prepared batches of dialyzed Bax. Scans were performed at a rate of 2°C/min, and Bax was used at a concentration of 13 µM. Control scans with lipid vesicles alone showed no transitions.

Membrane binding assay

Liposomes and Bax were co-incubated at a lipid to protein ratio of 500 for 3 hours at 37°C in targeting buffer. A fraction of this sample (120 µL) was adjusted to a final sucrose concentration of 1.4 M (final volume of 300 µL), overlaid with 400 µL of 0.8 M sucrose in buffer, and finally 300 µL of 0.25 M sucrose in buffer. Gradients were centrifuged at 400000 x g for 3 hours, and then four 250 µL fractions were drawn from the air-fluid interface. The top two fractions collected from the gradient correspond to the lipid-containing fractions and contain any membrane-associated Bax, while the bottom two fractions contain the remainder of the non-targeted protein. The polycarbonate centrifuge tubes were washed with 250 µL of hot 1% (w/v) SDS to give an indication of protein that had aggregated or adhered to the sides of the tube.

Gel filtration analysis of Bax oligomerization

Gel filtration chromatography was performed using a Superdex 200 HR 10/30 column from Amersham Biosciences equilibrated in 25 mM HEPES (pH 7.5), 300 mM NaCl, 0.2 mM dithiothreitol, and 2% (w/v) CHAPS. The sample was applied to the column in a total volume of 0.4 mL at a flow rate of 0.6 mL/min, and 0.4 mL fractions were collected (starting at the column void volume) and analyzed by Western blotting with anti-Bax antibody 2D2.

ANTS release assay for Bax pore formation

ANTS release from ANTS/DPX-charged liposomes was monitored by recording the dequenching of the ANTS fluorescence in a spectrofluorometer from Photon Technology
International, Inc. (Lawrenceville, NJ) using an excitation wavelength of 355 nm and monitoring the emission from 500 to 540 nm (slit widths set at 4 nm). Numerical values were obtained by integrating the area under the emission curve between 510 and 525 nm. The extent of ANTS release is reported as a percentage of the total determined by complete lysis of the liposomes with 0.2% Triton X-100. The emission maximum for ANTS-charged liposomes alone did not change over the duration of the assay, indicating that there was no spontaneous ANTS leakage. Likewise, the emission maximum for the detergent-lysed liposomes did not change over the duration of the assay, indicating that bleaching was not a factor. Cuvettes were incubated at 37°C, and cooled briefly to room temperature to take measurements. Bax and lipid were used at concentrations of 100 nM and 50 µM, respectively. The tBid and Bcl-X\textsubscript{L} proteins were used at concentrations of 5 nM and 200 nM, respectively. Final readings were taken after a 2 hour incubation, at which point the fluorescence intensity had reached a plateau.
RESULTS

Liposomes alone elicit a conformational change in Bax that can be detected by antibody 6A7

Monoclonal antibody 6A7 recognizes an epitope at the N-terminus of Bax that is occluded in its inactive state, but that becomes exposed upon Bax activation (30). Purified recombinant Bax does not react with 6A7 (Fig. 1A, lane 2), but can be artificially induced to adopt the activated, 6A7-positive conformation by incubation in buffer containing the detergent Triton X-100 (Fig. 1A, lane 1), as previously reported (30). Surprisingly, incubation of recombinant Bax with liposomes of mitochondrial outer membrane composition was also enough to trigger 6A7 epitope exposure (Fig. 1A, lane 3). This finding was somewhat unexpected—why then is the 6A7 epitope not constitutively accessible in whole cells where mitochondrial membranes abound? To address this question, immunoprecipitations were performed on samples of Bax incubated for 2 hours with purified mitochondria (Fig. 1B, lanes 1 and 2) or ER membranes (Fig. 1B, lanes 3 and 4). Membranes were lysed by the addition of either CHAPS or Triton X-100 (as a positive control) prior to 6A7 addition. Interestingly, neither mitochondria nor ER membranes caused the conformational change that was observed with liposomes alone. This conundrum is discussed in greater detail below.

As the pore-forming ability of Bax has recently been shown to depend upon the presence of the mitochondria-specific lipid cardiolipin (19,20), we next sought to determine whether individual lipids were also critical to inducing 6A7 epitope exposure. To this end, immunoprecipitations were performed on samples of Bax incubated with PG:PC (50:50 molar ratio) and PC only liposomes. Both types of liposome induced 6A7 epitope exposure (Fig. 1A, lanes 4 and 5), indicating that the change in Bax is not cardiolipin (or other lipid) specific. When lipid vesicles of the compositions tested above were lysed with CHAPS (a detergent that does
not affect Bax conformation) prior to mixing with the protein, they had no effect on Bax
conformation (Fig. 1A, lanes 6-8). Therefore the induction of Bax 6A7 epitope exposure depends
on an intact lipid vesicle, which cannot be substituted by the presence of lipids in mixed micelles
with detergent.

Since both PG:PC (net negative charge) and PC only (net neutral charge) liposomes
triggered the conformational change in Bax, the Bax-lipid interaction is likely not mediated by
electrostatic forces alone. To confirm this, immunoprecipitations were performed on samples of
Bax and liposomes co-incubated in targeting buffer supplemented with high salt (0.5 M NaCl) to
diminish electrostatic interactions. The higher salt conditions had no discernable effect (data not
shown), supporting the conclusion that the lipid-induced change in Bax is not solely due to
electrostatic interactions. Other documented properties of Bax activation include 1) a dependence
on the presence of Mg\(^{2+}\) (31) or Ca\(^{2+}\) (19) in some systems, and 2) inhibition of pore formation
by the membrane-active drug propanolol (32). Immunoprecipitations were performed to test
whether any of these variables affected the lipid vesicle-induced Bax conformational change, but
no divalent cation dependence or inhibition by propanolol was observed (data not shown).

Finally, to determine whether the Bax conformational change induced by lipid exposure
was a permanent or reversible event, protein and liposomes were co-incubated for a period of
two hours, and then protein was separated from liposomes by gel filtration using a column of
CL2B Sepharose. Control experiments demonstrated negligible binding of Bax to liposomes in
these experiments (data not shown, see also Fig. 4). The included fractions containing Bax
protein were pooled and divided into aliquots, and then 6A7 antibody was added at 0, 30, and 60
min post-separation, followed by immunoprecipitation. Bax that had not been exposed to
liposomes was also run over a CL2B column; pooled protein-containing fractions were divided
into two aliquots and treated with either CHAPS or Triton X-100 as negative and positive controls, respectively. The results are summarized in Fig. 1C, and show that the lipid-induced conformational change in Bax is rapidly reversible. Bax could not be immunoprecipitated with the 6A7 antibody once separated from liposomes, even when antibody was added immediately after separation (Fig. 1C, lane 3). Identical results were obtained when immunoprecipitations were repeated using samples in which protein and liposomes were separated by flotation (as per the Membrane binding assay in Experimental Procedures, data not shown). In summary, the liposome-induced exposure of the Bax 6A7 epitope 1) is reversible, 2) is not cardiolipin (or other lipid) specific, 3) is not mediated solely by electrostatic interactions, and 4) requires the presence of an intact lipid vesicle surface.

Characterization of the lipid-induced Bax conformational change by CD spectroscopy

All Bax-containing samples gave CD spectra with a predominantly $\alpha$-helical pattern (Fig. 2A). Deconvolution of the CD curves gave the following estimate of Bax secondary structure content: 66% $\alpha$-helix, 6% $\beta$-structure, 12% $\beta$-turns, and 16% random. These values agree well with those previously reported by NMR (26). In the presence of lipid, the secondary structure content was essentially unchanged: 67% $\alpha$-helix, 8% $\beta$-structure, 15% $\beta$-turns, and 10% random.

The thermal unfolding curves of Bax, obtained by recording the temperature dependence of the ellipticity at 222 nm, show an unfolding transition with partial loss of helicity occurring between 77°C and 88°C, with the midpoint of the transition region at 84-85°C (Fig. 2B). This transition is irreversible and results in a loss of approximately 40-50% of the original secondary structure. Scans performed after the heating cycle indicate that the remaining structure is still mostly helical in nature. The high denaturation temperature of Bax indicates that it is a protein.
with very stable structural organization; although Bax is mainly a cytoplasmic protein, it does not denature like soluble globular proteins, many of which unfold at around 65°C. In the presence of lipid, however, the unfolding transition of Bax was much smaller, broader, and partially reversible (Fig. 2C), with an approximate loss of only 20-30% of the original secondary structure. These data are consistent with the denaturation process having shifted to a higher temperature for a subset of Bax molecules. Therefore, CD spectroscopy shows that the structure of Bax acquires increased thermal stability in the presence of lipid vesicles, without undergoing any significant alterations in secondary structure.

**Characterization of the lipid-induced Bax conformational change by DSC**

The endotherms from the first heating cycle for Bax in the presence and absence of lipid vesicles are presented in Fig. 3. No further transitions were observed upon cooling or subsequent reheating. The unfolding transition for Bax alone appears at 79-80°C by DSC. (The slightly higher unfolding transition observed by CD spectroscopy is likely accounted for by the presence of glycerol in those samples, since glycerol is known to have a stabilizing effect on the structure of proteins (33)). In the presence of lipid vesicles, a second melting transition appears about 10-15°C higher than that for Bax alone, indicating that some Bax molecules adopt a more stable state as a result of the molecular changes initiated by the lipid vesicles. The exact spacing and resolution of the maxima for the two transitions in Fig. 3 varied somewhat from run to run (± 2°C, n=3), likely due to extrinsic factors such as the protein to lipid ratio, or the rate and mode of mixing. Nevertheless both the DSC results and the CD spectroscopy data demonstrate that the interaction of Bax with lipid vesicles causes a subset of Bax molecules to adopt a more stable conformation.
The lipid-induced Bax conformational change occurs without membrane insertion, oligomerization, or pore formation

To further examine the effect of liposome exposure on Bax conformation, flotation assays were performed to see if the lipid-induced conformational change in Bax resulted in membrane insertion. After co-incubation for 2 hours, liposomes and protein are layered at the bottom of a three-step sucrose gradient. Upon subsequent centrifugation, liposomes together with associated Bax float to the top of the gradient. Octyl glucoside-treated Bax was used as a positive control, as it displays all the characteristics of the activated protein including membrane insertion (27). The conformational change induced by incubation with liposomes does not result in Bax membrane insertion: Bax was not recovered from the top gradient fractions after incubation with liposomes, while there was significant targeting with the octyl glucoside-treated protein (Fig. 4).

Another key feature of Bax activation is the formation of large homooligomers. To test whether liposomes alone could induce the formation of Bax oligomers, both lipid-exposed and non-exposed protein were incubated for 2 hours at 37°C and subjected to gel filtration chromatography (Fig. 5A). The presence of 2% CHAPS in the column running buffer ensures the complete lysis of liposomes. Recombinant Bax runs as a monomer that elutes predominantly in fractions 20-24 (Fig. 5A). Bax that was pre-incubated with liposomes has essentially the same elution profile, but a very small amount is present in higher molecular weight fractions (from 14-18). However, octyl glucoside-treated Bax (used as the positive control) is completely shifted away from the monomeric form into much higher molecular weight complexes, as expected (27). Therefore, incubation of monomeric recombinant Bax with liposomes does not cause any significant oligomerization, consistent with previously published findings (20,34).
Finally, we tested the possibility that the lipid-induced conformational change in Bax induces pore formation. This scenario was highly unlikely based on the above data that liposomes do not induce Bax membrane insertion or oligomerization, however we felt it was a necessary experiment since the 6A7-positive conformation of Bax is so often associated with its pore-forming abilities. Incubation of Bax and liposomes did not result in significant ANTS release compared to the octyl glucoside-treated control, demonstrating that the lipid-induced exposure of the Bax 6A7 epitope is independent of pore-forming capability (see Fig. 5B). Therefore, although liposomes induce a conformational change in Bax that exposes the 6A7 epitope, this altered conformation of Bax does not undergo membrane insertion, oligomerization, or pore formation.

The Bax-lipid interaction is a prerequisite for tBid-induced Bax oligomerization and pore formation, and cannot be blocked by Bcl-XL.

To validate the biological relevance of the lipid-induced Bax conformational change, we sought to determine its significance to tBid-induced Bax activation, a well-characterized molecular pathway leading to apoptosis. First, since tBid is known to induce Bax oligomerization and pore formation, we co-incubated Bax and tBid and tested the oligomeric status of Bax both in the presence and absence of liposomes (Fig. 6A). tBid was only able to induce Bax oligomerization (and membrane permeabilization, see Fig. 6D) in the presence of liposomes, suggesting that the lipid-induced Bax conformational change must occur upstream of tBid activity. Significantly, tBid alone was also unable to induce the conformational change in Bax that is detected by the 6A7 monoclonal antibody (Fig. 6B, lane 4). However, Bax does become 6A7-reactive in the presence of both liposomes and tBid (Fig. 6B, lane 5), again suggesting that the lipid-induced exposure of the Bax 6A7 epitope occurs as a prerequisite to
tBid activity. To test whether the lipid-induced Bax conformational change might thus represent the step at which the anti-apoptotic protein Bcl-X\textsubscript{L} exerts its activity, immunoprecipitations were performed on samples of Bax co-incubated with liposomes and Bcl-X\textsubscript{L} (Fig. 6\textit{B}, lane 3). Since Bcl-X\textsubscript{L} did not block 6A7 epitope exposure, it can be concluded that Bcl-X\textsubscript{L} must act somewhere downstream of the Bax-lipid interaction to block Bax activation. The proteins used in these assays were verified to be pure by Coomassie Blue-staining of SDS-PAGE gels (Fig. 6\textit{C}), and verified to be functional by performing ANTS release assays for Bax-induced membrane permeabilization (Fig. 6\textit{D}). These data demonstrate that neither Bax nor tBid alone are sufficient to cause ANTS release from ANTS/DPX-charged liposomes, however the combination of the two leads to very efficient membrane permeabilization. The addition of anti-apoptotic Bcl-X\textsubscript{L} abrogates ANTS release, as predicted based on its anti-apoptotic function (Fig. 6\textit{D}).
DISCUSSION

Liposomes alone trigger a conformational change in Bax that results in 6A7 epitope exposure. CD spectroscopy and DSC both indicate that this lipid-induced conformational change causes Bax to adopt a different and more stable conformation. Yet the Bax 6A7 epitope is hidden in healthy cells, and only becomes exposed upon cellular injury or apoptotic stimulus. Indeed, our data show that mitochondria and ER membranes do not elicit the same changes in Bax as liposomes alone (Fig. 1B). How then can our observations be rationalized in terms of what is actually happening in cells?

One explanation for this puzzling scenario is likely the make-up of our in vitro system: highly-purified recombinant protein and liposomes. All other potential Bax-interacting proteins have been removed from the analysis, including all Bcl-2 family members, Ku70 and humanin (16,17), and any other unidentified players. Clearly, other proteins might interact with Bax to prevent the lipid-induced conformational change, or to sterically block epitope accessibility. Further, the membrane bilayer of a liposome does not exactly mirror the properties of cellular membranes. For example, biological membranes are protein-rich. If the trigger for the Bax conformational change involves a transient interaction with the hydrophobic bilayer interior or with a bilayer surface devoid of proteins (we have shown above that it is unlikely to be solely an electrostatic interaction), then areas of membrane damage or remodeling may facilitate Bax insertion into the membrane by priming the protein via the conformational change detected here. Arguing along these lines, Yamaguchi and Wang have observed that mitochondrial membranes that have been damaged by serial freeze-thaw cycles induce 6A7 epitope exposure in Bax, whereas undamaged mitochondria do not (35). Our results suggest a potential explanation for this observation: damage from the freeze-thaw cycles may expose breaches in the otherwise
stable membrane and allow transient interactions to occur with the bilayer, that induce 6A7 epitope exposure in similar fashion to liposomes.

Furthermore, an interesting observation has recently been made that Bax oligomerization only occurs at discrete foci on mitochondria, which subsequently become sites of mitochondrial fission (21). At these sites there are clearly alterations in the planar lamellar structure of the membrane and in the local resident protein content, both of which are likely contributing to the observed accumulation of Bax molecules. Perhaps the dynamic processes and membrane rearrangements occurring at these mitochondrial scission sites represent a biological equivalent to what is observed with our in vitro liposome system.

Interestingly, there is also a precedent in the literature for our observation that the Bax 6A7 epitope exposure is reversible upon removal of the inciting stimulus (in our case liposomes). Using a neuroblastoma-derived cell line, Dive and coworkers (36) showed that conformational changes at the N-terminus of Bax are rapidly induced by cytotoxic drug exposure or loss of cell-substrate interactions, and that these changes are fully reversible. Similarly, data from the Streuli lab (37) also show that Bax conformational changes encompassing the protein’s N-terminus can be reversible; in mouse mammary epithelial cells deprived of matrix attachment the Bax 62M epitope (amino acids 44-59) that is normally hidden becomes exposed, but upon matrix reattachment this epitope accessibility is once again lost. Notably, in both of these examples the conformational changes at the Bax N-terminus were not sufficient to commit the cells to apoptosis.

What then is the biological significance of Bax 6A7 epitope exposure? Using a defined system of recombinant proteins and liposomes, we have shown that the lipid-induced Bax conformational change is a prerequisite to tBid activity. tBid alone does not cause Bax 6A7
epitope exposure or oligomerization. However, when tBid and Bax are co-incubated in the presence of liposomes, Bax becomes 6A7-reactive and tBid is subsequently able to induce Bax oligomerization and pore formation (see Fig. 6). Therefore, one explanation is that the Bax N-terminal conformational change reflects an initial and essential step in the Bax activation process. In support of this interpretation, Shore and coworkers have previously dissected how changes at the Bax N- and C-termini affect the protein’s ability to target to intracellular membranes (4). They conclude that the C-terminal Bax α-helix is a signal-anchor required for membrane targeting and insertion, but that elements at the N-terminus are key to regulating this process. Deletion of the N-terminal 20 amino acids of Bax (encompassing the 6A7 epitope) stimulated Bax targeting to membranes in vitro and increased the pro-apoptotic activity of Bax in vivo (4). Perhaps exposure of the 6A7 epitope occurs when the N-terminus is released from some inhibitory interaction with the rest of the protein—one that may mask the C-terminal targeting sequence or a site of interaction with another protein. This hypothesis is consistent with our finding that 6A7 epitope exposure alone does not lead to Bax membrane insertion (see Fig. 4), but that a second factor is required to trigger insertion of the sensitized protein. We have shown that tBid can function as such a trigger factor for the sensitized Bax protein, but additional potential candidates include other pro-apoptotic Bcl-2 family members such as Bim (38), a mitochondrial-resident membrane protein (34), or Ca²⁺ (19).

Another important highlight of our data is that the lipid vesicle-induced exposure of the Bax 6A7 epitope does not depend on the presence of a specific lipid. At first glance, this observation may seem at odds with recent data demonstrating that the Bax-induced permeabilization of liposomes depends on the mitochondria-specific lipid cardiolipin (19,20).
However, cardiolipin has only been shown to be required for pore formation, prior steps in Bax activation have not been examined previously (19,20).

In summary, we have shown that Bax interacts transiently with lipid membrane surfaces where it undergoes a reversible conformational change that is not mediated solely by electrostatic forces or interactions with a specific lipid. The conformational change results in Bax adopting a more stable structure. It is not clear why this conformational change is rapidly reversible once lipid membranes are removed. Although exposure of the Bax 6A7 epitope has traditionally been associated with activation of the pro-apoptotic function of Bax, it more likely represents a sensitizing step that is one of the earliest stages of the Bax activation process in vivo. Indeed, we have shown that the lipid-induced exposure of the Bax 6A7 epitope is a prerequisite for tBid to induce Bax oligomerization and pore formation. We conclude that Bax activation can occur in discrete steps—a mechanism that provides opportunity for multiple levels of regulation.
ACKNOWLEDGMENTS

Dr. Richard Youle, NIH, generously provided antibodies to Bax. This work was supported by grants to D.W.A. and B.L., and to R.M.E., both from the Canadian Institutes of Health Research (CIHR). R.M.E. is a Senior Investigator of the CIHR. D.W.A. is the holder of a Canada Research Chair in Membrane Biogenesis. J.A.Y. is the recipient of a CIHR postdoctoral fellowship.
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FOOTNOTES

1 The abbreviations used are the following: ER, endoplasmic reticulum; PC,
phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS,
phosphatidylserine; DO, dioleoyl; ANTS, 8-aminonaphthalene 1,3,6-trisulfonic acid; DPX, p-
xylene-bis-pyridinium bromide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-
propanesulfonate; HEPES, N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid); CBD,
chitin-binding domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
CD, circular dichroism; DSC, differential scanning calorimetry; OG, octyl glucoside.
FIGURE LEGENDS

FIG. 1. Liposomes induce a conformational change in Bax that can be detected by immunoprecipitation with conformation-specific monoclonal antibody 6A7. A, Western blot of Bax immunoprecipitates after incubation of Bax with the following: lane 1, Triton X-100 (positive control); lane 2, CHAPS (negative control); lane 3, liposomes of mitochondrial outer membrane composition; lane 4, PG:PC (50:50) liposomes; lane 5, PC liposomes; lanes 6-8, as in lanes 3-5 except that liposomes were lysed with 2% CHAPS prior to incubation with Bax. B, Western blot of Bax immunoprecipitates after a 2 hour incubation of Bax with mitochondria (lanes 1 and 2) or ER (lanes 3 and 4) membranes. Immunoprecipitations were performed in buffer supplemented with 2% CHAPS (lanes 1 and 3), or 1% Triton X-100 (lanes 2 and 4, positive controls). C, Western blot of Bax immunoprecipitates to determine the reversibility of the lipid-induced Bax conformational change. Controls are shown in lane 1, Bax + Triton X-100 (positive control); and lane 2, Bax + CHAPS (negative control). Liposome-exposed Bax was separated from liposomes by CL2B gel filtration chromatography, and antibody 6A7 was added at the indicated times post separation; lane 3, t=0 min; lane 4, t=30 min; lane 5, t=60 min. *, binding of the secondary antibody to the light chain of antibody 6A7.

FIG. 2. Characterization of lipid-induced Bax conformational change by CD spectroscopy. A, CD spectrum of Bax in buffer, converted to mean molar residue ellipticity and expressed as deg cm$^2$/dmole. B, Thermal denaturation curve for Bax alone. C, Thermal denaturation curve for Bax in the presence of liposomes. Denaturation curves were obtained by recording the temperature dependence of Bax ellipticity at 222 nm. Temperature scans were performed at a heating or cooling rate of 1°C/min. Ellipticity was measured from 25°C to 95°C (lower data.
points), and then back to 25°C (higher data points) in order to assess the reversibility of

denaturation. The molar ellipticity at 25°C was set to -1 in order to compare the thermal
denaturation rates.

FIG. 3. **Characterization of the lipid-induced Bax conformational change by DSC.** DSC
curves for the thermal denaturation of Bax alone (lower curve) or Bax in the presence of
liposomes (upper curve). Scans were performed at a rate of 2°C/min. Curves are arbitrarily
displaced along the ordinate for visual presentation.

FIG. 4. **The lipid-induced Bax conformational change does not cause membrane insertion.**
Western blot of sucrose gradient fractions from Bax vesicle flotation assays (arrows indicate
decreasing sucrose concentration). Fraction 5 is obtained from a hot SDS wash of the emptied
centrifuge tube. Targeting reactions comprising Bax and liposomes of mitochondrial outer
membrane composition are loaded at the bottom of the gradient, in fraction 4. After
centrifugation, membrane-associated protein migrates into the upper fractions.

FIG. 5. **The lipid-induced Bax conformational change does not cause oligomerization or
pore formation.** A, Untreated Bax and Bax exposed to liposomes of mitochondrial outer
membrane composition or octyl glucoside were adjusted to 1% (w/v) CHAPS, and fractionated
on a Superdex 200 HR 10/30 gel filtration column. Fractions of 0.4 mL were collected starting at
the void volume, and every second fraction was analyzed by Western blotting with antibody
2D2. B, Bax membrane permeabilization was assessed by its ability to release ANTS from
liposomes of mitochondrial outer membrane lipid composition. Percent ANTS release values are
reported as the mean ± standard error (n = 3). Bax alone is incapable of ANTS release, even
though it adopts a 6A7-positive conformation under the given assay conditions. By contrast,
octyl glucoside-treated Bax (as a positive control) causes very efficient ANTS release from liposomes. Data were acquired after incubation for 2 hours.

FIG. 6. The lipid-induced Bax conformational change is required for tBid-induced Bax oligomerization, and is not inhibited by Bcl-XL. A, Untreated Bax and Bax exposed to tBid or tBid and liposomes were adjusted to 1% (w/v) CHAPS, and fractionated on a Superdex 200 HR 10/30 gel filtration column. Fractions of 0.4 mL were collected starting at the void volume, and every second fraction was analyzed by Western blotting with antibody 2D2. B, Western blot of Bax immunoprecipitates after incubation of Bax with the following: lane 1, CHAPS (negative control); lane 2, liposomes of mitochondrial outer membrane lipid composition (positive control); lane 3, Bcl-XL and liposomes; lane 4, tBid; lane 5, tBid and liposomes. *, binding of the secondary antibody to the light chain of antibody 6A7. C, Coomassie Blue-stained SDS-PAGE gel showing the purified recombinant proteins used in these assays: lane 1, molecular weight markers (118, 85, 48, 32, 26, and 19 kDa); lane 2, Bax; lane 3, tBid; and lane 4, Bcl-XL. D, Bax permeabilization of membranes was assessed by its ability to release ANTS from liposomes of mitochondrial outer membrane lipid composition. Reactions were incubated at 37°C, and briefly cooled to room temperature prior to taking fluorescence readings. Percent ANTS release values are reported as the mean ± standard error (n = 3). Bax (100 nM) or tBid (20 nM) alone do not release ANTS. By contrast, Bax and tBid together cause very efficient ANTS release. The addition of Bcl-XL (200 nM) is able to block the release of ANTS induced by the combination of tBid and Bax. Data were acquired after incubation for 2 hours.
Figure 1
Figure 2
Figure 3

Excess Heat Capacity (kJ/K/mol) vs. Temperature (°C) for Bax and Bax + lipid.
Figure 4
Figure 5
Figure 6
Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis
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J. Biol. Chem. published online September 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306289200

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