Membrane perturbations induced by the apoptotic Bax protein

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The apoptotic protein Bax, in oligomeric form, is effective in promoting both leakage and lipid mixing in liposomes composed of cardiolipin and phosphatidylethanolamine and/or phosphatidylcholine, upon the addition of calcium. In contrast, monomeric Bax is not active. At low concentrations at which caspase-8-cut Bid (tBid) alone has little effect on leakage, tBid augments the leakage caused by monomeric Bax. When solutions of oligomeric Bax are diluted to lower detergent concentrations than those required for Bax oligomerization, the protein is initially active in inducing liposomal leakage, indicating that the potency of the oligomeric form is not a consequence of being initially added to the liposomes in a high detergent concentration. However, in solutions of low detergent concentration, in the absence of liposomes, the oligomer gradually loses its lytic potency. This is accompanied by a loss of binding of bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonic acid), indicating the loss of exposed hydrophobic sites, as well as a loss of the ability of the protein to translocate to membranes. Membrane translocation was measured by an energy-transfer assay. It was demonstrated that membrane binding was greatly enhanced by oligomerization and by the presence of calcium. Thus the membrane-active form of Bax is unstable in the absence of detergent or lipid. In addition, we find that translocation to the membrane is enhanced by oligomerization as well as by the presence of high concentrations of calcium.

Key words: Bax inactivation, calcium promotion of leakage, hydrophobic binding site, membrane translocation.

INTRODUCTION

Bax, a member of the Bcl-2 family of proteins, plays an important role in promoting apoptosis in neurons [1]. Mitochondria play a central role in this process [2–4]. A characteristic feature of apoptosis is the rapid and complete release of cytochrome *c* from the mitochondria into the cytosol [5,6]. Bax promotes this pathway of apoptosis.

Bax is normally present in the cytosol, but translocates to the mitochondria during apoptosis [7–10]. Bax has been found to be present in oligomeric form in apoptotic cells, but mainly as a monomer in control cells [11]. The oligomeric form of Bax is required for channel-forming activity and for the release of cytochrome *c* from mitochondria [12–15]. A complex of only four Bax monomers is sufficient to form a channel that makes liposomes permeable to cytochrome *c* [13]. In addition, there is also evidence for the formation of large pores in mitochondria [16]. Electrical measurements in planar bilayers indicate that the channel formed by Bax is not like a conductance channel, but rather has variable size openings [17]. The oligomerization of Bax is promoted by caspase-8-cut Bid (tBid) [18]. However, the channels formed by this process are smaller than those observed with Bax oligomerized with detergent [19].

The importance of calcium in apoptosis has long been recognized. One aspect of this is the depletion of calcium stores in the endoplasmic reticulum during apoptosis [20]. There are close contacts between the endoplasmic reticulum and the mitochondria [21]. This probably facilitates the movement of calcium from the endoplasmic reticulum to the mitochondria [22,23]. In addition to its direct effect on mitochondrial permeability, Bax also has an independent function of promoting the movement of calcium from the endoplasmic reticulum to the mitochondria. This process is important to promote the release of cytochrome *c* from mitochondria [24,25]. The mechanism by which Bax stimulates this movement of calcium is not known. It is probably not a consequence of the pore-forming activity of this protein, since channel-conductance experiments indicate that it forms pores that are selective for monovalent anions [14]. These two functions of Bax may relate to the fact that cytochrome *c* is released from mitochondria by a two-step process [26].

In addition to the role of Bax in promoting the movement of calcium from the endoplasmic reticulum, this process is synergistic with other apoptotic actions of Bax on mitochondria [27], suggesting that increased calcium in the mitochondria promotes the translocation of Bax to the mitochondria and/or enhances the activity of Bax in causing leakage from the mitochondria. However, the situation is more complex, in that it has been shown that Bax is capable of stimulating cytochrome *c* release from mitochondria by both Ca^{2+}-independent, as well as Ca^{2+}-dependent, mechanisms [28]. Depletion of calcium with EGTA still allows for Bax to translocate to the mitochondria and to promote the release of cytochrome *c*, although this process becomes slower in the absence of calcium [10]. It is possible that calcium in the intermembrane space in mitochondria still plays a role in the action of Bax, even in the absence of cytosolic calcium.

The present work assesses the role of calcium and the conformational state of Bax in binding to membranes and altering membrane properties, including leakage and lipid mixing. The degree of exposure of hydrophobic sites on Bax under different conditions was measured as an indication of the ability of the protein to translocate to membranes. The probe 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonic acid (bis-ANS) has been used to bind to hydrophobic surfaces of proteins, where it undergoes an increase in quantum yield [29]. Mutational studies have demonstrated that the hydrophobic face of the amphipathic α-helical segment 2 is critical for both the dimerization and the apoptotic

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonic acid; DNS-PE, *N*-[5-(dimethylamino)naphthalene-1-sulphonyl]-L-α-phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; DPX, *p*-xylene-bis-pyridinium bromide; DTT, dithiothreitol; LUV, large unilamellar vesicle; tBid, caspase-8-cut Bid.

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activity of Bax [30]. Hydrophobic residues in this segment can be substituted with other hydrophobic residues, often without loss of function, while substitution of hydrophilic residues is more deleterious to function. Similarly, the protein functions of dimerization and promotion of apoptosis are less sensitive to substitution of the hydrophilic face of this helical segment than of the hydrophobic face [30]. Structural studies have confirmed the presence of this hydrophobic pocket and in addition this work showed that the hydrophobic sites can be covered by helical segment 9, suggesting that the orientation of helical segment 9 controls the dimerization and apoptotic functions of Bax [31]. Evidence for the existence of a hydrophobic groove in the anti-apoptotic protein, Bcl-2, was shown based on the enhancement of fluorescence of bis-ANS [32]. The hydrophobic bis-ANS-binding site was competed for with a Bak Bcl-2 homology 3 (BH3)-domain peptide. This method allows us to assess differences in the conformational properties of the monomer and oligomer in the presence and absence of calcium, independent of effects on the membrane. In addition, we have directly monitored membrane binding using a resonance-transfer assay between the Trp residues of Bax and the dansyl group of *N*-[5-(dimethylamino)naphthalene-1-sulphonyl]-L-α-phosphatidylethanolamine (DNS-PE).

EXPERIMENTAL

Materials

Full-length Bax with a tag of six histidines at the N-terminus was expressed in the pBAD plasmid of *Escherichia coli*; monomeric Bax was recovered in the soluble fraction and purified by chromatography on Ni^{2+}-nitrilotriacetic acid–agarose followed by Q-Sepharose as described previously [33]. It has been previously shown by us that the protein isolated in this manner is monomeric, but can be converted into an oligomeric form in the presence of octyl glucoside [15]. The stock solution of monomeric Bax was stored in 30% glycerol. The oligomeric Bax was maintained in that form with the presence of 1% octyl glucoside. The state of oligomerization of these preparations has been characterized using chemical cross-linking agents followed by Western blots. It was found that, in the absence of detergent, Bax has a molecular mass corresponding to a monomer and that in octyl glucoside the protein is oligomeric, with a mass of 140000 Da [15]. tBid was obtained from purified full-length Bid as described in [18]. tBid was in a solution of 15% glycerol with 0.5 mM EDTA. All lipids, including the fluorescently labelled lipids, were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The cardiolipin was a synthetic tetraoleoyl form. bis-ANS was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Preparation of large unilamellar vesicles (LUVs)

Lipids were dissolved in chloroform/methanol (2:1, v/v) at the desired molar ratio. The lipid was deposited as a film on the wall of a glass test tube by solvent evaporation with nitrogen. Final traces of solvent were removed for 2–3 h in a vacuum chamber attached to a liquid nitrogen trap. The lipid films were suspended in the appropriate buffer by vortexing at room temperature. The lipid suspensions were further processed with five cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA, U.S.A.) in a barrel extruder (Lipex Biomembranes, Vancouver, BC, Canada), at room temperature. LUVs were kept in ice and used within a few hours of preparation.

Liposomal content-leakage studies

Aqueous content leakage from liposomes was determined using the 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS)/*p*-xylene-bis-pyridinium bromide (DPX) assay [34]. Lipid films were hydrated with 12.5 mM ANTS, 45 mM DPX, 50 mM NaCl and 25 mM Hepes, pH 7.4. The osmolarity of this solution was adjusted to be equal to that of the buffer [50 mM NaCl, 0.2 mM dithiothreitol (DTT) and 25 mM Hepes, pH 7.5], as measured with a cryo-osmometer (Advanced Model 3MOplus Micro-Osmometer; Advanced Instruments, Norwood, MA, U.S.A.). The buffer was composed of 25 mM Hepes, 50 mM NaCl and 0.2 mM DTT, pH 7.5, adjusted to 300 mOsm. LUVs of 0.1 μm diameter were prepared by extrusion as described above. After passage through a 2.5 cm $\times$ 20 cm column of Sephadex G-75, the void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis. The fluorescence measurements were performed in 2 ml of buffer in a quartz cuvette equilibrated at 37 °C with stirring. Aliquots of LUVs were added to the cuvette to a final lipid concentration of 50 μM and the fluorescence was recorded as a function of time using an excitation wavelength of 360 nm and an emission wavelength of 530 nm with 8 nm bandwidths. A 490 nm-cut-off filter was placed in the emission path. One of the proteins in buffer was added to the lipid vesicles in the cuvette to give a final protein concentration in the cuvette of 20 nM. Leakage was initiated by the addition of several microlitres of a 1 M $CaCl_2$ solution in buffer. The amount of Ca^{2+} added was chosen so as to cause only slow leakage from the LUV alone. The rate of leakage caused by the proteins was measured over several minutes. The value for 100% leakage was obtained by adding 20 μl of a 10% Triton X-100 solution to the cuvette. Runs were done in duplicate. The LUVs were composed of dioleoylphosphatidylcholine (DOPC)/cardiolipin (2:1, molar ratio), dioleoylphosphatidylethanolamine (DOPE)/cardiolipin (2:1, molar ratio) or DOPC/DOPE/cardiolipin (1:1:1, molar ratio). The Bax oligomer was added from a solution of 1% octyl glucoside that is required to induce the formation of oligomers of this protein [15]. However, the final concentration of octyl glucoside in the cuvette was only 0.00025%, and this concentration was shown not to affect liposomal leakage. Some protein solutions also contained glycerol, but appropriate controls demonstrated that the glycerol had no effect on leakage at the final concentrations used.

Lipid-mixing assay for membrane fusion

The resonance-energy-transfer assay of Struck et al. [35] was used to monitor membrane fusion. This assay is based on the dilution of a pair of fluorescently labelled lipids into a larger amount of unlabelled lipid. This results in a decrease in the efficiency of energy transfer between the fluorescent probes. LUVs were prepared containing either DOPC/cardiolipin or DOPE/cardiolipin at a molar ratio of 2:1 or a mixture of equimolar amounts of DOPC/DOPE/cardiolipin. For each of these lipid systems two populations of LUVs were prepared, one unlabelled and one labelled with 2 mol% each of *N*-(lissamine Rhodamine B sulphonyl)phosphatidylethanolamine and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine. A 9:1 molar ratio of unlabelled to labelled liposomes was used in the assay. Fluorescence was recorded at excitation and emission wavelengths of 465 nm and 595 nm, respectively; using a 490 nm-cut-off filter placed between the cuvette and the emission monochromator, with 8 nm bandwidths, using an SLM Aminco Bowman AB-2 spectrofluorimeter. Siliconized glass

cuvettes (1 cm^2) were used with continuous stirring in a thermostat-controlled cuvette holder. Measurements were carried out using a buffer containing 25 mM Hepes, 50 mM NaCl and 0.2 mM DTT, pH 7.5. LUVs at a final lipid concentration of 50 μM were added to 2 ml of buffer in the cuvette at 37 °C and then the protein was injected to give a final concentration of 20 nM. Lipid mixing was initiated by addition of the indicated amount of $CaCl_2$ solution. The amount of Ca^{2+} added was such that it caused only a slow rate of lipid mixing with the LUV alone.

The effect of the proteins on the rate of lipid mixing was monitored. Fluorescence was recorded for several minutes and then 20 μl of 10 % Triton X-100 was added (final concentration, 0.1 %). The initial residual fluorescence intensity prior to addition of calcium, F_0, was taken as zero. The maximum fluorescence intensity, F_{max}, was obtained by dilution of the labelled lipids with 20 μl of 10 % Triton X-100. The percentage of lipid mixing at time t is given by $[(F_t - F_0)/(F_{max} - F_0)] \times 100$. All runs were done in duplicate and were found to be in close agreement. Appropriate controls were done that demonstrated that the glycerol or the octyl glucoside, which were present in some of the protein samples, had no effect on lipid mixing at the final concentrations used in the assays.

bis-ANS fluorescence

A solution of bis-ANS was added to 2 ml of a buffer of 50 mM NaCl, 0.2 mM DTT and 25 mM Hepes, pH 7.5, to give a final concentration of 6 μM bis-ANS. Bax, either in monomeric form or as an oligomer in octyl glucoside solution, was added at a concentration of 50 nM Bax to the buffer prior to the addition of bis-ANS at different times after incubation. In the case of the oligomer, the final octyl glucoside concentration in the cuvette was 0.0005 %. Fluorescence was measured using siliconized glass cuvettes, at 37 °C. Excitation was set at 400 nm and the emission spectra recorded five times and averaged. A 4 nm bandwidth was set for excitation and emission. A 420 nm-cut-off filter was placed between the cuvette and the emission monochromator. Time dependence of the emission spectrum was then determined.

Binding of Bax to membranes

The resonance-energy-transfer assay between the Trp residues of Bax and the dansyl group on DNS-PE was used to assess the translocation of the protein to a membrane. LUVs were prepared containing DOPC/DOPE/cardiolipin (1:1:1 molar ratio) with 5 mol % DNS-PE added. The LUVs were diluted to a concentration of 10 μM and the fluorescence emission measured using an excitation wavelength of 290 nm. The change of emission intensity at 520 nm was recorded as a function of time before and after the addition of 6 mM Ca^{2+} and/or 50 nM Bax, in either monomeric or oligomeric form, at 37 °C. In the case of the oligomer, the final octyl glucoside concentration in the cuvette was 0.0005 %.

RESULTS

Leakage

Leakage from vesicles is strongly promoted by the oligomeric form of Bax, but not by monomeric Bax (Figure 1). The lipid dependence of the leakage rates was compared using liposomal mixtures composed of DOPC/DOPE/cardiolipin (1:1:1) and dioleoylphosphatidylglycerol (DOPG)/DOPC/DOPE (1:1:1). Leakage was initiated by the addition of 6 mM Ca^{2+}. Vesicles

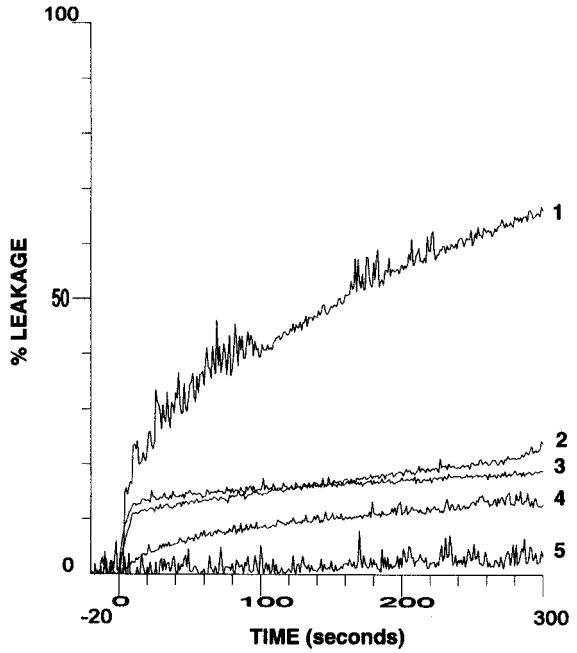

Figure 1 Effect of Bax on the rate of leakage of ANTS and DPX from liposomes of DOPC/DOPE/cardiolipin and DOPC/DOPE/DOPG in molar ratios of 1:1:1

The lipid concentration was 50 μM and the protein 20 nM. Leakage was initiated by addition of 6 mM calcium at zero time. Trace 1, Bax oligomer added to LUVs of DOPC/DOPE/cardiolipin. Trace 2, Bax monomer added to LUVs of DOPC/DOPE/cardiolipin. Trace 3, LUVs of DOPC/DOPE/cardiolipin. Trace 4, Bax oligomer added to LUVs of DOPC/DOPE/DOPG. Trace 5, LUVs of DOPC/DOPE/DOPG.

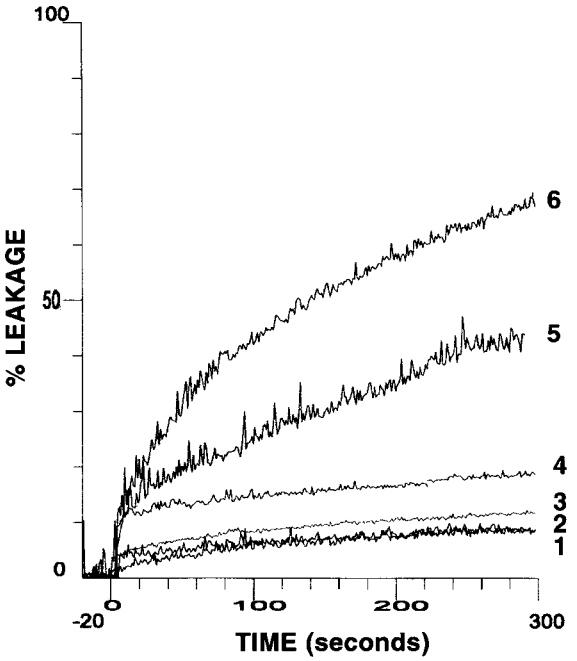

Figure 2 Effect of calcium concentration on the rate of leakage of ANTS and DPX from liposomes of 50 μM DOPC/DOPE/cardiolipin (1:1:1) and 20 nM Bax oligomer

Leakage was initiated by addition of the concentration of calcium (in mM) indicated at the right-hand side of the curve.

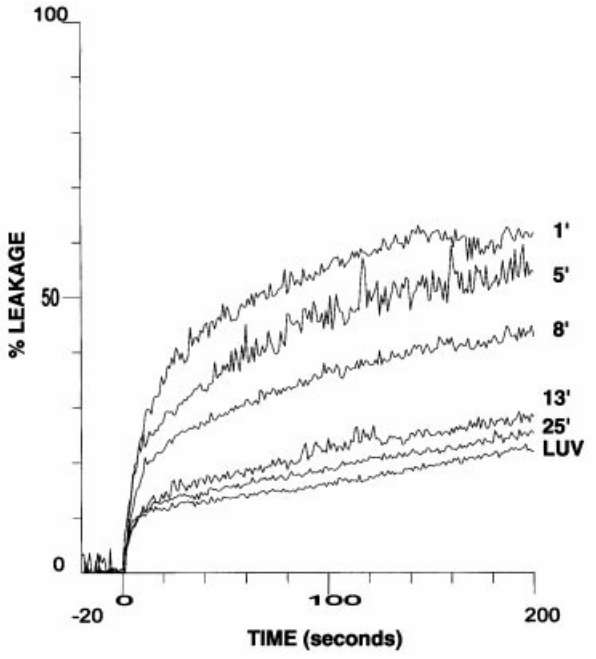

Figure 3 Loss of leakage inducing potency of 20 nM Bax oligomer upon incubation in buffer at various times, in the absence of liposomes, at 37 °C

Leakage was measured with 50 μM LUVs of DOPC/DOPE/cardiolipin (1 : 1 : 1) upon the addition of 6 mM Ca^{2+}. LUVs were added at the indicated times (1–25 min). Similar loss of potency was observed in lipid-mixing assays.

containing cardiolipin in mixtures with other lipids have different sensitivity to leakage induced by calcium. For each liposome system a calcium concentration was chosen so that, in the absence of protein, the liposomes would not exhibit significant release of entrapped probes, but at the same time the concentration of calcium used was close to that required to induce leakage in the liposomes without protein. Leakage was negligible in the absence of calcium. For all of the systems only low rates of leakage were observed with the Bax monomer. However, the Bax oligomer induced significant fast leakage from liposomes containing cardiolipin (Figure 1). There is an initial small burst of leakage, followed by a more gradual process. The initial burst is likely to be a consequence of a high local concentration of Bax at the site of addition before complete mixing takes place. We compare the rate of leakage subsequent to this initial burst. The leakage was relatively insensitive to the presence of phosphatidylethanolamine or phosphatidylcholine (results not shown), unlike the case of tBid, which is sensitive to membrane curvature [36]. Substituting DOPG for cardiolipin significantly reduced the rate of leakage induced by Bax (compared at the same molar concentrations as those used in the cardiolipin-containing mixtures), although, unlike with tBid, leakage with the DOPG-containing liposomes could still be detected with the Bax oligomer (Figure 1). Thus while it is likely that curvature plays a role in the effects of Bax on liposomes, the relationship does not exhibit a strong correlation as in the case of tBid, and it may depend on the specific nature of the lipid system.

The sensitivity of leakage to the presence of Ca^{2+} was determined (Figure 2). Although the rate of leakage rose with increasing concentrations of Ca^{2+} between 1 and 6 mM, it did not approach rapid rates until concentrations of 5 or 6 mM were reached.

We tested whether the difference in leakage rates between the Bax monomer and oligomer could be a consequence of the presentation of the protein in octyl glucoside solution compared with detergent-free buffer. Using the oligomer in octyl glucoside solution, we reversed the addition of solutions to the cuvette, adding the protein before the liposomes. With this protocol the

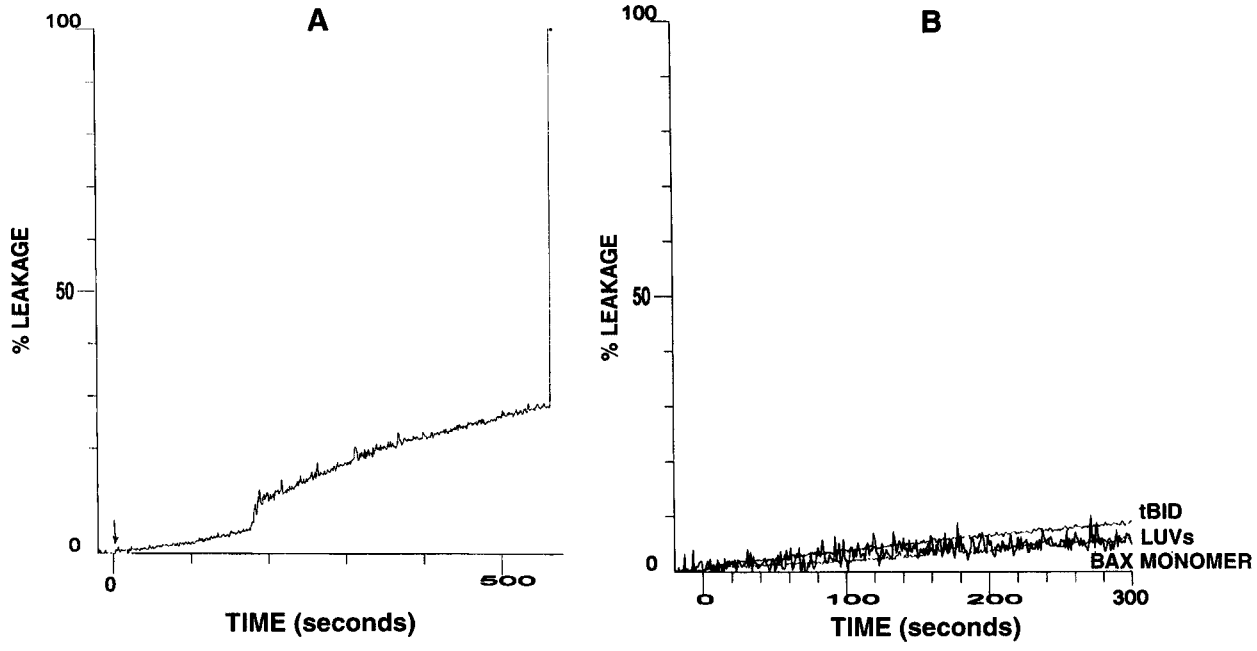

Figure 4 Promotion of vesicle leakage from 50 μM liposomes composed of DOPC/cardiolipin (2:1) with 8 mM $CaCl_2$

(**A**) tBid (4 nM) mixed together with 20 nM Bax monomer and added to liposomes. (**B**) Control with 4 nM tBid, 20 nM Bax monomer or LUVs. The arrow indicates the initiation of leakage upon addition of Ca^{2+}.

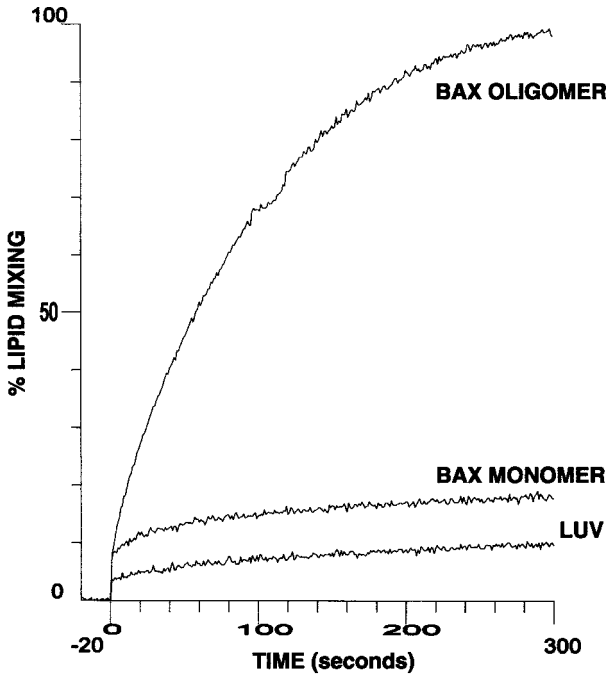

Figure 5 Lipid mixing promoted by Bax with liposomes of DOPC/DOPE/cardiolipin (1:1:1) at 5 mM Ca^{2+}

The total lipid concentration was 50 μM and Bax was present at 20 nM, as either the monomer or oligomer. In addition to the control of LUVs alone (LUVs), LUVs in the presence of Bax monomer and oligomer are shown.

oligomer bound to the liposomes in a solution that had a very low detergent concentration. In contrast, when the protein in detergent is added last, before mixing is complete, the protein may encounter some lipid while it is still surrounded by a high detergent concentration. The amount of leakage obtained was independent of the order of addition. These results indicate that the greater leakage-promoting potency of the Bax oligomer is a property of the protein and is not an artifact of the detergent. In addition, if the oligomer is incubated in the cuvette in the absence of lipid, it loses its ability to promote leakage with a half time of several minutes (Figure 3). A similar time-dependent loss of potency was observed using a lipid-mixing assay (results not shown).

Combinations of Bax monomer and tBid were tested for their effects on liposomal leakage. Using liposomes of DOPC/cardiolipin (2:1) there was enhanced leakage caused by Bax monomer in the presence of low concentrations of tBid. At these low concentrations, tBid by itself has almost no effect on leakage (Figure 4). A short lag phase was observed in this system before leakage commenced, which may correspond to the time required for tBid to promote the oligomerization of Bax [18].

Lipid mixing

As with leakage, lipid mixing is promoted by the oligomeric form of Bax, but not by the Bax monomer (Figure 5). The curves shown are measured with liposomes of DOPC/DOPE/cardiolipin (1:1:1), but there is little sensitivity of lipid mixing induced by oligomeric Bax to lipid composition when comparing DOPC/cardiolipin (2:1) or DOPE/cardiolipin (2:1). The monomeric form of Bax was considered to be inactive in perturbing membranes. However, although this protein has weak activity, it showed some weak lipid mixing activity with DOPE/cardiolipin (2:1) liposomes (results not shown).

Table 1 Time-dependent changes in the fluorescence of bis-ANS in the presence of Bax

Fluorescence intensity is shown, relative to the fluorescence of the Bax oligomer at 1 min, measured at the emission maximum (492 nm for the oligomer and 496 nm for the monomer). All measurements were made in the absence of calcium. Values are corrected for the fluorescence of bis-ANS in buffer in the absence of protein.

	Fluorescence intensity	
Time (min)	Monomer	Oligomer
1	0.35	1
10	0.2	0.64
20	0.18	0.4
30	Not determined	0.27

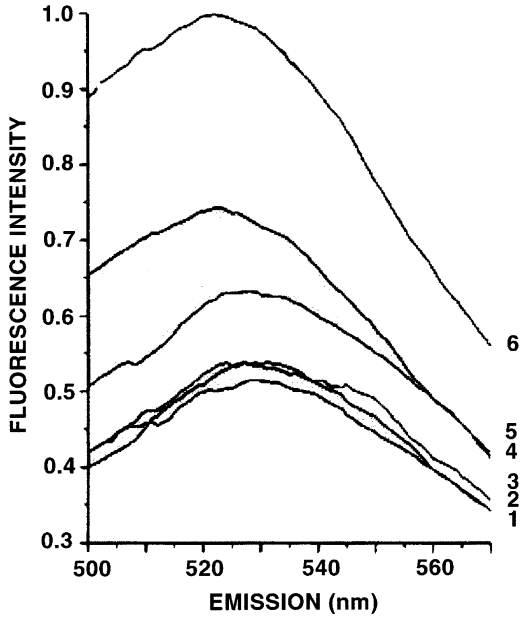

Figure 6 Binding of Bax to membranes by fluorescence-energy-transfer assay

LUVs (10 μM) of equimolar DOPC/DOPE/cardiolipin containing 5% DNS-PE were mixed with 50 nM protein with or without 6 mM Ca^{2+}. Greater fluorescence intensity is indicative of increased protein translocation to the membrane. Trace 1, Bax monomer; trace 2, control without Ca^{2+} (no protein added); trace 3, control with Ca^{2+} (no protein added); trace 4, Bax oligomer; trace 5, Bax monomer with Ca^{2+}; trace 6, Bax oligomer with Ca^{2+}.

bis-ANS binding

Bax is found largely in the cytosol of cells not undergoing apoptosis [7–10]. In order to translocate to the membranes of intracellular organelles, Bax must undergo a conformational change to expose hydrophobic groups. The increased fluorescence of bis-ANS is a useful probe for the exposure of hydrophobic sites and allows for the comparison of the protein's conformational states independent of any differences in the membrane. The fluorescence of bis-ANS was measured in the presence of Bax monomer and Bax oligomer. The oligomer was formed in

octyl glucoside solution as described previously [15]. The fluorescence of the bis-ANS in the presence of Bax oligomer was measured as a function of time after the essential removal of detergent by dilution to an octyl glucoside concentration of 0.00125 %. Controls showed that this concentration of detergent itself had no effect on the fluorescence of bis-ANS. It is clear that the oligomer bound more bis-ANS than the monomer of Bax (Table 1). There was a progressive decrease in bis-ANS fluorescence with time (Table 1). This loss of exposure of hydrophobic sites is in accord with our finding that the leakage potency of the protein decreases with time after dilution (Figure 3).

Membrane binding of Bax

Measurement of the efficiency of resonance-energy transfer from the Trp residue to the dansyl group of DNS-PE has been used to study the partitioning of peptides and proteins to membranes. The Bax monomer has little affinity for membranes but there is a smaller degree of translocation of the monomer to the membrane in the presence of Ca^{2+} (Figure 6). In contrast, the Bax oligomer has a moderate affinity with membranes that is considerably augmented by the presence of calcium (Figure 6).

DISCUSSION

Calcium increases the rate and extent of Bax-promoted leakage or lipid mixing obtained with the cardiolipin-containing liposomes assayed in this work. In the presence of calcium, with this lipid system it is possible to observe rapid leakage using protein concentrations in the nanomolar range. The increased amount of calcium in mitochondria is important to promote the release of cytochrome *c* [24,25]. The increased potency of Bax in the presence of calcium, observed in the present study, mimics that observed with mitochondria. The concentration of calcium required for this effect is several millimolar (Figure 2) and therefore higher than levels attained in the cytoplasm. However, the local concentration of calcium at the interface of anionic membranes, such as the inner mitochondrial membrane, can reach these values. In addition, after initiation of the process of apoptosis, it has been shown that certain caspases cleave and inactivate the plasma membrane Ca^{2+} pump, resulting in increased cytosolic levels of Ca^{2+} [37]. The principle effect of calcium is not on the conformation of the protein, which results in a relatively small increase in bis-ANS fluorescence (results not shown), but rather on the membrane. Calcium is known to induce membrane destabilization of cardiolipin-containing liposomes [38]. In addition, the α-isoform of Bax used in this work [39] has an isoelectric point of 5.08. Therefore at neutral pH the protein will be negatively charged, as will the cardiolipin-containing membrane. Calcium binding to the electrical double layer at the membrane interface will screen the charge repulsion between protein and membrane and thus facilitate the translocation of the protein to the membrane surface.

Perturbation of the membrane still requires the oligomeric form of Bax. Monomeric Bax, even in the presence of calcium and at concentrations comparable with those used for the oligomeric form, does not cause either vesicle leakage (Figure 1) or extensive lipid mixing (Figure 5). The ability of Bax to promote lipid mixing may facilitate access of the protein to cardiolipin present in the inner mitochondrial membrane. Cardiolipin facilitates leakage promoted by Bax to a much greater extent than a structurally related lipid, phosphatidylglycerol (Figure 1). This may be a consequence of the cardiolipin, not phosphatidylglycerol, membranes becoming destabilized in the presence of calcium [38,40–42].

A factor that may contribute to the membrane-perturbing activity of Bax is its ability to translocate to membranes. In cells Bax is found to be largely in the cytosol [7–9], presumably in a monomeric state. The inability of the monomer to bind to membranes (Figure 6) is consistent with these findings. The protein appears to translocate to membranes under conditions in which it exposes hydrophobic groups, as assessed by bis-ANS binding (Table 1). Thus there is greater exposure of hydrophobic groups in the oligomer compared with the monomer and therefore the oligomer has a greater binding affinity for membranes. The oligomer exhibits a greater exposure of hydrophobic groups and has greater partitioning to membranes, in both the presence and absence of calcium, than does the monomeric form of Bax.

Both the Bax monomer and oligomer lose the exposure of hydrophobic groups when incubated in buffer at 37 °C. The loss of exposed hydrophobic groups in the oligomer, after dilution of the octyl glucoside to low concentrations, is accompanied by a loss of the ability to promote leakage (Figure 3). This could be a result of a slow dissociation of the oligomer or an aggregation of the exposed hydrophobic sites leading to denaturation of the protein.

It has also been shown that tBid promotes the oligomerization of Bax [18]. In agreement with this, we found that low concentrations of tBid, which by itself has little effect on liposome properties, can slowly promote the release of liposomal contents caused by the Bax monomer (Figure 3). There is evidence that in cells Bax (or Bak) must be present together with tBid in order to promote apoptosis. In the cell tBid is N-myristoylated and targeted to the mitochondria [43]. Neither Bax in the absence of Bid [44] nor tBid in the absence of Bax [45] can induce apoptosis *in vivo*. However, *in vitro* with isolated mitochondria, Bax promotion of the release of cytochrome *c* is independent of the presence of tBid, and together Bax and tBid are capable of acting synergistically [46]. These findings can be explained in terms of the membrane-destabilizing actions of tBid in the mitochondria *in vivo* being enhanced by the increased mitochondrial calcium concentration that is promoted by Bax [24,25]. Bax also binds directly to the mitochondria and may also have direct actions on this organelle, independent of its role in the movement of calcium. The increased potency of Bax in the presence of tBid may be a result of tBid-promoted oligomerization of Bax leading to greater membrane leakage, analogous to what we observe with liposomes (Figure 3).

Our work confirms the importance of Bax oligomerization for its membrane-perturbing activity. Furthermore it demonstrates that the oligomer has more exposure of hydrophobic groups and a greater tendency to translocate to a membrane than does the monomeric form of Bax. The membrane affinity, as well as the ability of Bax to induce leakage in cardiolipin-containing membranes, is augmented in the presence of calcium.

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