The apoptotic protein tBid promotes leakage by altering membrane curvature.

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The apoptotic protein tBid is effective in promoting both leakage and lipid mixing in liposomes composed of cardiolipin and phosphatidylcholine at a molar ratio of 1:2 in the presence of calcium. When half of the phosphatidylcholine component of these liposomes is replaced with phosphatidylethanolamine, a lipid that promotes negative membrane curvature, the rates of both leakage and lipid mixing caused by tBid are substantially increased. Replacement of cardiolipin with phosphatidylglycerol, a lipid that is structurally similar to cardiolipin but does not promote negative membrane curvature in the presence of calcium, prevents the tBid from promoting leakage. The promotion of leakage by tBid is also inhibited by several substances that promote positive membrane curvature, including lysophosphatidylcholine, tritrypticin, a potent antimicrobial peptide, and cyclosporin A, a known inhibitor of cytochrome c release from mitochondria. We directly measured the effect of tBid on membrane curvature by 31P NMR. We found that tBid promotes the formation of highly curved non-lamellar phases. All of these data are consistent with the hypothesis that tBid promotes negative curvature, and as a result it destabilizes bilayer membranes. Bcl-XL inhibits leakage and lipid mixing induced by tBid. Bcl-XL is anti-apoptotic. It reduces the promotion of non-bilayer phases by tBid, although by itself Bcl-XL is capable of promoting their formation. Bcl-XL has little effect on liposomal integrity. Our results suggest that the anti-apoptotic activity of Bcl-XL is not a consequence of its interaction with membranes, but rather with other proteins, such as tBid.

The Bcl-2 family of proteins plays an important role in regulating the intracellular apoptotic signal cascade (1, 2). Members of this protein family can be either pro-apoptotic, such as tBid, or anti-apoptotic, such as Bcl-XL. Mitochondria play a central role in this process (3–5). A characteristic feature of apoptosis is the rapid and complete release of cytochrome c from the mitochondria into the cytosol (6, 7).

One of the pro-apoptotic Bcl-2 proteins is Bid. Unlike other Bcl-2 proteins that have several domains, Bid has only one BH3 domain. The proteolytic enzymes, the caspases, also play an important role in apoptosis. One of the roles of caspases-8 is to catalyze the cleavage of Bid to a truncated form, tBid (8–10). The tBid is then N-myristoylated, which results in its targeting to mitochondria (11) where it releases cytochrome c and other proteins from the intermembranous compartment. Cardiolipin is required for the binding of t-Bid to mitochondria (12).

Several mechanisms have been demonstrated for the action of tBid. It has been shown that tBid can promote the oligomerization of both Bak (13, 14) as well as Bax (15). The oligomerization of Bak and Bax is required for their pro-apoptotic activity (16). However, it has recently been shown that tBid itself homo-oligomerizes, and this process, without the participation of Bak or Bax, results in the release of cytochrome c (17) as well as other proteins (18) from mitochondria. In addition, it has been observed that tBid promotes leakage in model membranes in the absence of other proteins (19, 20).

A physical property of the membrane that will contribute to its stability is the intrinsic curvature of its constituent monolayers (21). Cell membranes are essentially flat structures on a molecular scale. However, each monolayer of the bilayer may have greater stability after acquiring certain degree of curvature. Minimum curvature energy will be reached when the monolayer bends to shape equal to its intrinsic curvature. When a bilayer has a large intrinsic negative monolayer curvature, it will spontaneously convert from a flat structure to an inverted phase, such as the hexagonal phase. In the hexagonal phase the phospholipids are arranged as cylinders with an aqueous core. The cylinders are packed with hexagonal symmetry, and the phospholipids are oriented with their headgroups adjacent to the aqueous core. Membrane monolayer curvature may have particular importance in relation to the functioning of mitochondria. It is known that mitochondrial lipids will convert from a lamellar to a hexagonal phase in the presence of Ca2+ (22, 23). Freeze fracture electron microscopy has also demonstrated the presence of non-bilayer structures in intact mitochondria (24). The propensity of the mitochondrial membrane to form hexagonal phases has been suggested to modulate the movement of calcium through the membrane (25) as well as the activity of certain mitochondrial enzymes (26).

Interestingly, it is known that cytochrome c itself will induce the formation of the hexagonal phase in bilayers of cardiolipin (27), one of the major lipid components of the inner mitochondrial membrane.

In addition to the release of cytochrome c across the outer mitochondrial membrane, it is also found that tBid induces a striking remodeling of mitochondrial structure with a mobilization of about 85% of the cytochrome c stores in cristae (28). During this process, individual cristae become fused and the...
Membrane Curvature and tBid Function

The morphological reorganization of mitochondria may also be related to the propensity of the membrane to form inverted phases. It has been shown that the membranes of mitochondria of amoeba form structures resembling a lipidic cubic phase, a structure formed by membranes with negative curvature propensity (29). The morphological reorganization of mitochondria observed during apoptosis is inhibited by cyclosporin A (28), a peptide that has been shown to inhibit membrane processes requiring increased negative curvature (30). Furthermore, ceramides, a class of lipids that promotes negative curvature (31), cause the release of cytochrome c from isolated mitochondria (32–34). Ceramides have also been recently shown to form stable conductance channels in membrane bilayers (35). Together these observations are highly suggestive that monolayer curvature properties modulate mitochondrial function and that increased negative membrane curvature is associated with apoptosis.

There are several ways by which the promotion of leakage can be related to intrinsic monolayer curvature. There is evidence for the formation of a highly curved, non-lamellar phase in mitochondria, i.e., the cubic phase (36). An even more highly curved lipid phase is the hexagonal phase. Formation of this phase will completely break down the permeability barrier of the mitochondria. This is a rather extreme consequence and would result in the release of all materials from the mitochondria. However, even without undergoing a phase transition, simply a change in the propensity to form a phase with a highly curved morphology would be sufficient to destabilize the bilayer and allow more rapid release of mitochondrial contents. This leakage would occur, as has been observed with tBid (20), without the formation of channels. Alternatively, leakage may occur through a protein pore whose formation or transport activity could be increased by greater intrinsic negative curvature in the bilayer. Such curvature modulation of transport has been observed, for example, with the ionophores alamethicin (37) and gramicidin (38).

Cardiolipin may be important for the actions of Bcl-2 proteins and their relationship to membrane curvature. When the negative charge of this lipid is neutralized by Ca\(^{2+}\), the lipid readily converts from a bilayer to a highly curved non-bilayer phase, the hexagonal phase (39). Interestingly, Bax and Bak promote the movement of Ca\(^{2+}\) from the endoplasmic reticulum to the mitochondria (40, 41). In addition, cardiolipin may be important for the binding of certain Bcl-2 proteins, as has been suggested for tBid (12). However, cardiolipin is a lipid component of the inner mitochondrial membrane, whereas Bcl-2 proteins bind to the outer mitochondrial membrane (15). Nevertheless, cardiolipin may interact with the Bcl-2 proteins as a consequence of protein-promoted lipid transfer. Increased rates of lipid transfer have been shown with both Bid and tBid (42). There is evidence that components of the permeability transition pore reside at contact points between the inner and outer membranes of mitochondria (43). It has been shown that tBid also localizes to mitochondria at these contact sites (44). The marked morphological changes in mitochondria that are induced by tBid strongly suggest that the inner mitochondrial membrane is also involved in the apoptotic action of this protein (28).

We have further elucidated how tBid interacts with membranes and how the lipid composition of membranes affects the actions of this protein. We have studied the leakage of vesicle contents, a process related to the release of proteins such as cytochrome c and Smac/DIABLO from the intermembrane space. We have also measured the propagation of lipid mixing induced by tBid, a process that may be similar to that which allows lipids from the inner mitochondrial membrane to transfer to the outer membrane. Leakage and lipid mixing are two functional manifestations of membrane destabilization, and the extent of one of these processes may be altered at the expense of the other (45). The liposomes we used in this study contained cardiolipin and the stability of the cardiolipin-containing bilayer was decreased by the addition of calcium. We evaluated the role of the intrinsic curvature properties of the liposome by substituting phosphatidylethanolamine (PE), a lipid that forms stable bilayers, for phosphatidylethanolamine (PE), a lipid that promotes negative intrinsic monolayer curvature. Insertion of a protein that promotes negative curvature should be more disruptive to a bilayer that already has a negative curvature tendency because of the presence of PE. We also measured effects of tBid on intrinsic membrane curvature by directly monitoring the modulation of the polymorphism of a lipid mixture, using \(^{31}\)P NMR. The shape of the \(^{31}\)P NMR spectrum is known to be different for lipids in the lamellar phase and in non-bilayer phases such as the inverted hexagonal phase (46). As the temperature is increased the acyl chains of phospholipids in bilayers will acquire increased splay. This will increase the negative curvature tendency of the membrane, and, at sufficiently high temperature, this will convert the lipid to the hexagonal phase. We measured the modulation of the relative amounts of bilayer and hexagonal phases by tBid as a function of temperature as well as a function of lipid to protein ratio. In addition, we present evidence that several agents that promote positive curvature inhibit tBid-promoted vesicle leakage as well as the release of cytochrome c from isolated mitochondria.

**EXPERIMENTAL PROCEDURES**

**Materials—Caspase-8-cut Bid (tBid)** was obtained from purified full-length Bid as described in a previous study (15). The resulting N- and C-terminal fragments were not separated. It has been found that the mixture of the two fragments has comparable activity to the isolated C-terminal fragment (20). A 10-fold excess of the N-terminal is required to inhibit the action of the C-terminal fragment in the presence of membranes. tBid was kept as a stock solutions in 15% glycerol with 0.5 mM EDTA. Bcl-X\(_{L}\) was prepared as previously described (47). The stock solutions of Bcl-X\(_{L}\) were in 30% glycerol. The tritritcin was a synthetic product purified by high-performance liquid chromatography and obtained from Professor Vogel at the University of Calgary. Cyclosporin A was purchased from Sigma Chemical Corp. (St. Louis, MO). All lipids, including the fluorescently labeled lipids, were purchased from Avanti Polar Lipids (Alabaster, AL). The cardiolipin was a synthetic tetradecyl form and the lysophosphatidylcholine (LPC) was the 1-palmitoyl form.

**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 h in a vacuum chamber attached to a liquid nitrogen trap. The lipid film was hydrated with 12.5 mM ANTS, 45 mM DPX, 50 mM NaCl, 25 mM triethanolamine (lissamine rhodamine B sulfonylphosphatidylethanolamine; N-Rh-PE; N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; DTT, dithiothreitol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bis-pyridinium bromide.

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1 The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; N-Rh-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; LUV, large unilamellar vesicle; MLV, multilamellar vesicles; DTT, dithiothreitol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bis-pyridinium bromide.
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 DTT, 25 mM HEPES, pH 7.4) as measured with a cryo-osmometer (Advanced Model 3MPlus Micro-Osmometer, Advanced Instruments Inc., Norwood, MA). LUVs of 0.1-μm diameter were prepared by extrusion as described above. After passage through a 300-μm diameter Sephacryl G-75, the concentrated 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 emission wavelength of 530 nm with 8-nm bandwidths. A 490-nm cutoff 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 with the addition of several microliters of a 1 mM CaCl2 solution in buffer. The amount of Ca2+ added was chosen so as to cause only slow leakage from the LUV alone. The potentiation or inhibition 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 DOPC:cardiolipin (2:1) or DOPC:DOPE:cardiolipin (1:1:1). The solution of tBid contained glycerol, but appropriate controls demonstrated that the glycerol had no effect on leakage at the final concentrations used in the assays.

Lipid Mixing Assay for Membrane Fusion—The resonance energy transfer assay of Struck et al. (49) was used to monitor membrane fusion. LUVs were prepared containing either DOPC:cardiolipin (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 unlabeled and one labeled with 2 mol% each of N-Rh-PE and N-NBD-PE. A 9:1 molar ratio of unlabeled to labeled liposomes was used in the assay. Fluorescence was recorded at excitation and emission wavelengths of 465 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 spectrophotometer. Siliconized glass cuvettes (1 cm) were used with continuous stirring in a thermostatted 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 CaCl2 solution. The amount of Ca2+ added was chosen 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, F0, was taken as zero. The maximum fluorescence intensity, Fmax, was obtained by dilution of the labeled lipids with 20 μl of 10% Triton X-100. Peroxidase-mediated lipid mixing at time t is given by ∆F = (Fmax − F0)/(Fmax − F0) × 100. All runs were done in duplicate and were found to be in close agreement. Appropriate controls were done that demonstrated that the glycerol that was present in the stock solution of tBid had no effect on lipid mixing at the final concentrations used in the assays.

Centrifugation Assay for Membrane Binding of tBid—A dried lipid, prepared as described above, was hydrated by suspending in buffer by vortexing at room temperature, followed by three cycles of freezing and thawing. An aliquot of MLVs was then taken into buffer or protein solution containing calcium. These mixtures were freeze-thawed three times and then incubated for 60 min at 37 °C. Vesicles were pelleted by centrifugation at 200,000 × g for 150 min at 25 °C. The supernatant was removed and assayed for protein.

Determination of Phospholipid Concentration—The concentration of phospholipid was determined by measuring the amount of inorganic phosphate released after digestion by the method of Ames (50).

Determination of the Concentration of Protein—The concentration of protein was determined by the CBQCA assay (Molecular Probes) and read after 3 h of incubation at room temperature, in a 96-well plate with a SpectraMax microplate spectrophotometer equipped with SPECTRAmax Pro software. Appropriate lipid controls were carried out for all assays.

31P NMR—The 31P NMR spectra were measured using suspensions containing 5 mg of a mixture of DOPC:DOPE:cardiolipin (1:1:1). The lipid mixture was first deposited as a film from a solution in chloroform/methanol (2:1, v/v) as described above for the preparation of LUVs. The lipid film was hydrated by vortexing with 25 mM HEPES, 50 mM NaCl, 0.2 mM DTT, pH 7.5 in the absence or presence of tBid and/or Bcl-XL.

FIG. 1. Effect of tBid on the leakage of ANTS and DPX from 50 μM liposomes of cardiolipin:DOPC (1:2 molar ratio) with 8 mM Ca2+ (CL/PC); cardiolipin:DOPC:DOPE (1:1:1 molar ratio) (CL/PC/PE) or DOPG:DOPC:DOPE (1:1:1 molar ratio) (PG/PC/PE) with 6 mM Ca2+ (CL/PC/PE) in the presence and absence of 20 nM tBid (control liposomes of DOPG:DOPC:DOPE a) show little leakage but are not shown). In all figures of leakage or lipid mixing, the process was initiated by addition of appropriate amounts of calcium at zero time.

After the lipid was suspended at room temperature, a small volume of CaCl2 solution was added to make the calcium concentration 10 mM. The suspension was then frozen and thawed three times to assure equilibration, and the sample was loaded into a 5-mm diameter Shigemi NMR tube (Shigemi Co., Tokyo, Japan). Controls with lipid alone showed that glycerol had no effect on the lipid polymorphism. Spectra were obtained using a Bruker AM-500 spectrometer operating at 202.45 MHz in a 10-mm broadband probe over a 50-KHz sweep width in 16 × 1024 data points. A 90° pulse width of 16.6 μs was used. Composite pulse decoupling was used to remove any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. Probe temperature was maintained to ±0.2 °C by a Bruker B-VT 1000 variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR magnet.

Cytochrome c Release—Mitochondria from HeLa cells have been prepared by differential centrifugation as previously described (51). Mitochondria (30 μg of protein) were incubated with various concentrations of tritritpin in the presence or absence of 5 μM tBid for 20 min at room temperature in 100 μl of KCl buffer (125 mM KCl, 4 mM MgCl2, 5 mM phosphate, 0.5 mM EGTA, 5 mM succinate, 15 mM Hepes-KOH, pH 7.4). Mitochondria were then centrifuged for 5 min at 13,000 × g, and both the pellets and supernatants were analyzed for cytochrome c by Western blot. Equal loading of the mitochondrial pellet was verified using an antibody against prohibitin.

RESULTS

Leakage—Leakage of vesicle contents is strongly promoted by tBid (Fig. 1). We have compared the amount of leakage from LUV composed of DOPC:cardiolipin (2:1 molar ratio) with vesicles of DOPG:DOPE:cardiolipin (1:1:1 molar ratio). In both cases, leakage was initiated by the addition of ~7 mM Ca2+. A calcium concentration was chosen so that in the absence of protein the liposomes would not exhibit significant leakage, but at the same time the concentration of calcium used was close to that required to induce leakage in the liposomes without protein. The relative leakage rates were sensitive to the lipid composition. In particular, tBid became more potent with liposomes containing PE (Fig. 1). Liposomal leakage promoted by tBid has recently been shown in another study in which the
The importance of the presence of anionic lipids was demonstrated (19). The earlier results cannot be directly compared with our data, because they were done using liposomal systems that did not contain cardiolipin and calcium was not added.

The promotion of leakage caused by tBid as a result of substituting DOPC with DOPE, suggests that the increase in the lytic action of tBid in the presence of PE may be a consequence of the fact that this lipid promotes negative curvature. To further test this we introduced substances that promoted positive membrane curvature and would be expected to inhibit the lytic action of tBid. We tested tritritpticin, cyclosporin A, and LPC, substances of very different chemical nature, all of which promote positive membrane curvature. One of these was tritritpticin, an antimicrobial peptide (52), and another was the lipid LPC (53, 54). Each of these substances was added to 50 μM LUV of DOPC:cardiolipin (2:1) or DOPC:DOPE:cardiolipin (1:1:1) to give a final concentration of additive of 7 μM. After the addition of Ca$^{2+}$ and 40 nM tBid (twice the concentration used to obtain the data of Fig. 1), leakage reached a value of 10% or less of the total contents, over the first 200 s. We also tested cyclosporin A, an immunosuppressive peptide that inhibits processes requiring negative curvature (30). At a concentration of 10 μM this peptide completely inhibited leakage from DOPG/DOPC/DOPE (1:1:1). Thus several different positive curvature agents strongly inhibit the action of tBid.

Cardiolipin forms non-lamellar structures in the presence of calcium but diolceylphosphatidylglycerol (DOPG) does not. We therefore substituted DOPG for cardiolipin in liposomes with DOPG/DOPC (2:1) (not shown) or DOPG/DOPC/DOPE (1:1:1) (Fig. 1). Liposomal leakage is almost completely inhibited when
DOPG is substituted for cardiolipin.

One of the reasons for the difference in lytic activity of tBid with vesicles of different lipid composition could be a consequence of different degrees of binding to these different liposomal mixtures. To measure binding of tBid to lipid we used MLV to more easily separate the soluble and lipid-bound protein. In addition, an increased concentration of protein was used to be able to detect the protein that remains in the supernatant more accurately. We find that in the presence of Ca\(^{2+}\) about half of the tBid binds to MLV of either DOPC:cardiolipin (2:1) or DOPC:DOPE:cardiolipin (1:1:1) (Fig. 2). The relatively small difference in the binding between these two lipid systems cannot explain the severalfold difference observed in leakage rates with liposomes of the two different lipid compositions (Fig. 1). Replacing cardiolipin with DOPG actually increases the binding of tBid to the liposomes (Fig. 2). Hence, the lack of leakage activity of tBid with DOPG-containing liposomes is not a consequence of the inability of the protein to bind.

We have also tested combinations of pro- and anti-apoptotic Bcl-2 proteins on liposomal leakage. Bcl-X\(_L\) has little effect on contents leakage from liposomes of DOPC:cardiolipin (2:1) (Fig. 3) nor from liposomes of DOPC:DOPE:cardiolipin (1:1:1) even
after addition of calcium (not shown). There is a large inhibition of the leakage induced by tBid upon the addition of Bcl-XL (Fig. 3).

**Lipid Mixing**—As with leakage, lipid mixing is promoted by tBid using liposomes containing cardiolipin in the presence of calcium (Fig. 4). In addition, as with leakage, lipid mixing promoted by tBid is greater using liposomes of DOPC:DOPE:cardiolipin (1:1:1) compared with those of DOPC:cardiolipin (2:1). However, the sensitivity to lipid composition is smaller with lipid mixing (Fig. 4) than was seen with leakage (Fig. 1). As with liposome leakage (Fig. 3), there is a large inhibition of the lipid mixing induced by tBid with the addition of Bcl-XL (Fig. 5).

**31P NMR**—The morphology, and hence phase, of a lipid aggregate can be assessed by 31P NMR. It is known that the addition of calcium will promote the hexagonal phase (55) and membrane fusion (56, 57) with liposomes containing cardiolipin. We have measured the thermally induced polymorphic transition of a mixture of DOPC:DOPE:cardiolipin (1:1:1) with 10 mM CaCl2 in the presence and absence of tBid (Fig. 6). Because of the limited amount of protein available, to maintain a lipid to protein molar ratio of about 2500, we had to use only 5 mg of the lipid sample. At 20 °C in the absence of protein, the 31P NMR spectrum had a peak upfield of phosphoric acid, which was set of 0 ppm (Fig. 6). By 40 °C the major peak has shifted downfield below the chemical shift of phosphoric acid. However, at 40 °C there is still significant intensity in the powder pattern of the pure lipid mixture above 0 ppm that dies away at higher temperature. The spectra indicate that there is a gradual shifting from bilayer to hexagonal phase for the pure lipid as the temperature increases from 20 to 40 °C, but at 50 °C or higher, the hexagonal phase is the predominant one. tBid promotes the formation of considerable hexagonal phase even at 20 °C, and by 40 °C the conversion is virtually complete (Fig. 6). The stock solution of this protein contained 15% glycerol. The addition of 30% glycerol to the lipid controls results in the incomplete conversion to the hexagonal phase at higher temperature. Thus, at the lower concentrations of glycerol used, it does not completely prevent the formation of the hexagonal phase by tBid. At 10-fold higher concentration of tBid (with 15% glycerol), there is a strong promotion of the formation of the hexagonal phase and there is virtually complete conversion at 20 °C (Fig. 7). Surprisingly, Bcl-XL, even though it has opposite biological properties to tBid, also strongly promotes the hexagonal phase (Fig. 8). However, a combination of Bcl-XL and tBid promotes less hexagonal phase than either component alone (Fig. 8).

**Cytochrome c Release**—Tritrpticin, a peptide that is known to promote positive membrane curvature (52), was tested for its ability to modify Bax-induced cytochrome c release. Mitochondria from HeLa cells were used in these experiments. Bax was previously shown to be attached to mitochondria from these cells following their isolation. Addition of tBid to mitochondria from HeLa cells results in Bax insertion and oligomerization and finally leads to cytochrome c release (15). Tritrpticin was tested at concentrations ranging from 0.1 to 100 μM for its ability to counteract the action of Bax. Controls made with the addition of 10 or 100 μM tritrpticin to mitochondria led to a small release of cytochrome c. This peptide is known to have potent bactericidal activity (52). As with many other peptides of its class, at higher concentrations it is lytic to liposomes2 as well as to mitochondria. However, at lower concentrations no significant release of cytochrome c from mitochondria was detected. As expected, addition of tBid to mitochondria was accompanied by cytochrome c release. This effect was completely inhibited by the concomitant addition of 100 μM tritrpticin. At lower concentrations, tritrpticin had no significant effect.

**DISCUSSION**

There are several results that suggest that destabilization of membrane bilayers by increasing the intrinsic negative curvature of the membrane favors apoptosis. Calcium strongly promotes negative curvature in membranes containing cardiolipin (39). Certain pro-apoptotic Bcl-2 proteins cause the movement of calcium into the mitochondria (40, 41, 58). In addition, in cells exposed to the pro-apoptotic agent, staurosporin, cyto-
chrome c release is promoted by Ca\(^{2+}\) uptake into the mitochondria (40, 41, 59). It is known that negative curvature in membranes decreases with decreasing temperature. An increased lag time and decreased rate of cytochrome c release from mitochondria induced by tBid is observed with a relatively small decrease in temperature from 37 to 25 °C (6). This observation is also consistent with the protein being less efficient when there is less negative curvature strain in the membrane. Cyclosporin A, an inhibitor of processes requiring increased negative curvature in membranes (30), inhibits apoptosis (28). Ceramides are well known as promoters of apoptosis (32–34) and these lipids promote negative monolayer curvature (31).

In this report we present several additional lines of evidence to indicate that tBid alone can destabilize phospholipid bilayers. In addition, this destabilization is, at least in part, a consequence of tBid promoting negative curvature in membrane bilayers. In accord with this relationship, leakage of liposomal contents promoted by tBid is considerably more rapid from vesicles with greater negative intrinsic monolayer curvature, in which DOPE is substituted for half of the DOPC (Fig. 1). This is also the case for lipid mixing, although with less sensitivity to lipid composition (Fig. 4). Furthermore, three structurally unrelated substances, tritritpticin, cyclosporin A, and LPC, which promote positive curvature, inhibit liposomal leakage promoted by tBid. In addition, substitution of cardiolipin by DOPG prevents leakage induced by tBid (Fig. 1). This is also in accord with curvature properties, because cardiolipin acquires negative curvature in the presence of Ca\(^{2+}\), whereas DOPG does not. More directly, we show that tBid promotes the formation of a highly curved non-bilayer phase, the hexagonal phase, compared with lipid alone, both as a function of temperature (Fig. 6) as well as of tBid concentration (Fig. 7).

In addition to the action of tBid alone, likely through homoolimerization (17), tBid can also affect the oligomerization of other Bcl-2 proteins. It has also been shown that tBid promotes the oligomerization of Bax (15). There is evidence that in cells Bax (or Bak) must be present together with tBid to promote apoptosis. Neither Bax in the absence of Bid (60) nor tBid in the absence of Bax (61) can induce apoptosis in vivo. However, in vitro with isolated mitochondria, Bax promotes the release of cytochrome c independent of the presence of tBid and together Bax and tBid acted synergistically (62). These findings can be explained in terms of the membrane-desestabilizing actions of tBid in the mitochondria in vivo requiring the increased mitochondrial calcium concentration that is promoted by Bax (40, 41). In the cell tBid is N-myristoylated and targeted to the mitochondria (11). In addition to its action in promoting movement of calcium to the mitochondria, Bax also binds directly to the mitochondria and may also have direct actions on this organelle. Just as there are many pathways to apoptosis, there may also be more than one mechanism by which each of the pro-apoptotic proteins acts. Thus, there are two possible mechanisms by which the bilayer-stabilizing tritritpticin can inhibit tBid-promoted leakage of cytochrome c from mitochondria. One is by a direct inhibition of the action of tBid on mitochondria and the other is through inhibition of tBid-promoted oligomerization of Bax. In either case, the results support the conclusion that membrane curvature modulates the apoptotic action of tBid.

There is evidence that the anti-apoptotic protein, Bcl-X\(_L\), also forms complexes with Bcl-2 proteins containing a BH3 domain (63). In agreement with this, Bcl-X\(_L\) inhibits both leakage (Fig. 3) and lipid mixing (Fig. 5) promoted by tBid. This protein also inhibits the promotion of the hexagonal phase formation by tBid (Fig. 8). However, by itself, Bcl-X\(_L\) is capable of promoting the formation of the hexagonal phase. This would not be expected for a protein that is anti-apoptotic and does not promote vesicle leakage or lipid mixing. Bcl-X\(_L\) lacks the C-terminal domain that anchors the Bcl-2 proteins into the outer membrane of the mitochondria. However, at high concentrations helices 5 and 6 of Bcl-X\(_L\) could insert into the membrane as a result of a conformational change. It has been shown that cleaved forms of Bcl-X\(_L\) can induce membrane leakage (64). It is possible that the segments of Bcl-X\(_L\) responsible for this can insert into membranes to promote formation of the hexagonal phase. The ability of Bcl-X\(_L\) to inhibit the action of tBid may thus be a consequence of protein-protein interaction between Bcl-X\(_L\) and tBid (63), rather than through opposing modulation of membrane properties.

In summary, our studies show that tBid increases negative curvature strain in membranes and promotes the formation of non-bilayer inverted phases. Negative curvature strain is well correlated with the ability of tBid to efficiently induce membrane leakage, both with regard to the promotion of liposomal leakage by DOPE as well as its inhibition by tritritpticin, cyclosporin A, and LPC. The bilayer destabilization caused by tBid is facilitated by the increased calcium concentration in the mitochondria, which is known to promote negative curvature with cardiolipin and to occur during apoptosis. In addition, we directly demonstrate, using \(^{31}P\) NMR, the effect of tBid on lipid polymorphism. tBid also interacts with other Bcl-2 proteins. Apoptosis is a complex process in which several proteins and many factors enter into play. In this report we demonstrate that the properties of the membrane also play an important role in this complex process.

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