Transbilayer Lipid Diffusion Promoted by Bax: Implications for Apoptosis†

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ABSTRACT: It is known that the proapoptotic protein Bax facilitates the formation of pores in bilayers, resulting in the release of proteins from the intermitochondrial space. We demonstrate that another consequence of the interaction of Bax with membranes is an increase in the rate of lipid transbilayer diffusion. We use two independent assays for transbilayer diffusion, one involving the formation of asymmetric liposomes by placing a pyrene-labeled lipid into the outer monolayer of preformed vesicles and another assay based on the initial preparation of liposomes having an asymmetric transbilayer distribution of lipids. With both methods we find that oligomeric BaxΔC or full-length Bax in the presence of tBid, but not monomeric full-length Bax, strongly promotes the rate of transbilayer diffusion. Although biological membranes exhibit rates of lipid transbilayer diffusion of minutes or less, they are able to maintain an asymmetric distribution of lipids across the bilayer. In the case of mitochondria, cardiolipin is sequestered on the inner leaflet of the inner mitochondrial membrane. However, during apoptosis this lipid translocates to the outer surface of the outer mitochondrial membrane. This phenomenon must involve an increase in the rate of transbilayer diffusion. The results of the present paper demonstrate that an activated form of Bax can cause this increased rate.

The importance of the transbilayer asymmetric distribution of lipids in biological membranes has long been recognized. To attain a more accurate understanding of biological membranes, one has to take into account not only the bilayer and its constituent proteins but also the asymmetry of the lipid distribution between the two leaflets of the bilayer (*1*). Not only does transbilayer asymmetry have general biological importance, but also it has particular roles in apoptosis (*2*).

The release of proteins from the intermitochondrial space is a major step in the proapoptotic action of Bax (*3*−*10*). There is evidence that cardiolipin is important in apoptosis (*10*−*13*). The major fraction of cardiolipin in cells is present on the inner leaflet of the inner membrane of the mitochondria (*14*), although some cardiolipin has been reported to be present in the outer mitochondrial membrane (*15*, *16*) and at contact sites where inner and outer membranes appear to join (*17*, *18*). An early event in apoptosis is that cardiolipin translocates from the inner to the outer monolayer of the inner mitochondrial membrane (*19*, *20*). Cardiolipin exhibits a rapid rate of transbilayer internalization in the inner mitochondrial membrane, on the order of minutes or less (*21*). Thus, to account for the appearance of cardiolipin on the outer monolayer of the inner mitochondrial membrane, there must be an increase in the rate of transbilayer diffusion of this lipid. The mechanism resulting in the acceleration of phospholipid transbilayer diffusion in mitochondria is not known. In addition to the increased rate of transbilayer diffusion in the inner mitochondrial membrane, cardiolipin must also become exposed to the outer surface of the outer mitochondrial membrane to maximally induce apoptosis. It has been shown, at least with some forms of apoptosis, that there is increased exposure of cardiolipin at the surface of the mitochondria during this process (*16*). For cardiolipin to become exposed on the surface of the mitochondria, it must undergo exchange from the inner mitochondrial membrane to outer membrane and must undergo transbilayer diffusion in each of the mitochondrial membranes.

In the present study we demonstrate that Bax is capable of promoting the transbilayer diffusion of lipids. There have been many studies of Bax-induced leakage using model liposomal systems (*4*, *11*, *22*, *23*) demonstrating that Bax promotes the formation of pores in bilayers. Small peptides that promote leakage of vesicle contents also promote transbilayer diffusion of lipids (*24*) as do transmembrane peptides (*25*). The acceleration of the rate of transbilayer diffusion concomitant with an increased rate of leakage is indicative of the formation of a membrane pore that is lined with both lipid and protein (*26*).

It is well established that the rate of transbilayer diffusion of lipids is much greater in biological membranes than in liposomes. In addition, there are proteins that specifically stimulate this process. In particular, mitochondria contain a phospholipid scramblase that would accelerate movement of phospholipids between monolayers (*27*). However, the action of scramblase is not sufficiently rapid to overcome other processes that maintain phospholipid transbilayer asymmetry in normal cells. Little if any cardiolipin is found on the surface of mitochondria (*28*). Cardiolipin has been shown,

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however, to appear on the mitochondrial surface during apoptosis (*16*, *20*). Therefore, to account for this, there must be some additional process or stimulation of an existing pathway to increase the rate of transbilayer diffusion of this lipid. The simplest and most direct mechanism would be for a proapoptotic protein to directly stimulate this process. We demonstrate the feasibility of this mechanism in the present work. In analogy to the properties of antimicrobial peptides, the finding that there is transbilayer movement of phospholipid together with leakage of solutes across the bilayer indicates that Bax forms pores in the membranes that are lined with both lipid and protein.

MATERIALS AND METHODS

Materials. Human Bax-α lacking 20 amino acids at the carboxyl terminus, BaxΔC,[1] was produced as previously described (*29*). The purified protein was stored in 30% glycerol, 25 mM Hepes, 0.2 mM DTT. A 10-fold dilution of this solution into 10 mM Hepes, 0.14 M NaCl, 0.1 mM EDTA was made prior to use. This form of the protein is oligomeric and active in causing pore formation in membranes without the addition of detergent. 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-nitrilotriacetic acid agarose followed by Q-Sepharose. Caspase8 cut Bid (tBid) was obtained from purified full-length Bid as described previously (*30*). tBid was kept as a stock solution in 15% glycerol with 0.5 mM EDTA. Prior to taking small aliquots into the cuvette, this solution was diluted 10-fold as done with BaxΔC. The pyrene-labeled lipid 1-lauroyl-2-(1′-pyrenebutyroyl)-*sn*-glycero-3-phosphocholine (py-12-PC) was a generous gift from Drs. Andreas Herrmann and Peter Müller from Humboldt University in Berlin. 1-Hexadecanoyl-2-(1′-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (py-16-PC), *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (DNS-DHPE), and Oregon Green−dextran (70 kDa) conjugate were purchased from Molecular Probes (Eugene, OR). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL). The cardiolipin (CL) was a synthetic tetraoleoyl 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 2−3 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 to form multilamellar vesicles. 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) in a barrel extruder (Lipex Biomembranes, Vancouver, BC), at room temperature. LUVs were kept on ice under nitrogen and used immediately after preparation.

Rate of Lipid Transbilayer Diffusion Using Extruded Vesicles. We measured the rate of transbilayer lipid diffusion (flip-flop) using a method described by Müller et al. (*24*). The method is based on the dilution of the pyrene probe py-12-PC as a result of transbilayer diffusion. The probe stock was made by dissolving it in ethanol and then adding it to PBS (pH 7.2) to a final ethanol concentration of 5%. An aliquot of this stock solution of the probe was then added to a final concentration of 0.5 μM in 2 mL of buffer in a quartz cuvette. A suspension of LUV in PBS, pH 7.2, was then added to give a final concentration of 10 μM. This results in the probe partitioning only into the outer monolayer at a concentration of 5 mol % of the total lipid or 10 mol % of the outer monolayer. The emission intensity of pyrene for the excimer and for the monomer fluorescence was continuously measured at two different wavelengths, and the ratio of these intensities was recorded as a function of time. The excitation wavelength was 344 nm, with a 4 nm bandwidth. The fluorescence of the monomer was measured at an emission wavelength of 395 nm and at 477 nm for the excimer. The fluorescence ratio was followed until a plateau was reached after the addition of the LUVs. A positive control with melittin was also run and gave results very similar to those previously reported (*24*). When flip-flop occurs in the presence of BaxΔC, there is a reduction in the excimer-to-monomer ratio because of the dilution of the probe from one monolayer to two. The observed I_e/I_m was used to calculate the extent of flip-flop following the procedure of Müller et al. (*24*). The dependence of I_e/I_m on the fraction of probe that has undergone transbilayer diffusion is calculated from a calibration curve as a function of the mole percent of pyrene. The details of this calculation have been described (*24*). Controls for effects on the fluorescence that were independent of flip-flop were also run using symmetrically labeled LUVs. These symmetrically labeled LUVs were prepared by extrusion as described above, with the pyrene-labeled lipids mixed with the other lipids in organic solvent prior to making the lipid film and hydrating. Measurements were done with two independent preparations. Values of the ratio I_e/I_m were normalized to that of the LUVs alone and then averaged.

Binding of Bcl-2 Proteins to Membranes. The resonance energy transfer assay between the Trp residues of BaxΔC, full-length monomeric Bax, or tBid and the dansyl group on DNS-DHPE was used to assess the translocation of the protein to a membrane. LUVs were prepared with 5 mol % DNS-DHPE added. The LUVs were diluted to a concentration of 50 μM in a quartz cuvette containing 2 mL of buffer. Vesicles were used immediately after extrusion. The buffer used was an EGTA−calcium buffer containing 10 mM Hepes, 0.14 M NaCl, pH 7.4, adjusted to contain 100 nM *free* calcium; 1 mM Mg^{2+} was added after addition of LUVs and proteins. This condition reproduces those under which leakage was measured. The fluorescence was measured at 37 °C using an excitation wavelength of 280 nm, with a 90° polarizer in the excitation path and a 0° polarizer in the

[1] Abbreviations: BaxΔC, human Bax-α lacking 20 amino acids at the carboxyl terminus; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; PI, soybean phosphatidylinositol; CL, tetraoleoylcardiolipin; DOPS, dioleoylphosphatidylserine; LUV, large unilamellar vesicle; py-12-PC, 1-lauroyl-2-(1′-pyrenebutyroyl)-*sn*-glycero-3-phosphocholine; py-16-PC, 1-hexadecanoyl-2-(1′-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; DNS-DHPE, *N*-dansyldihexadecanoylphosphatidylethanolamine; QUELS, quasi-elastic light scattering; I_e, fluorescence emission intensity of the pyrene excimer; I_m, fluorescence emission intensity of the pyrene monomer.

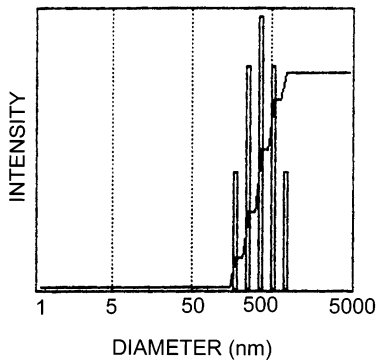

FIGURE 1: Typical size distribution of asymmetric liposomes, determined by QUELS.

emission path. The emission spectra were recorded three times and averaged, before and after the addition of 100 nM BaxΔC or full-length Bax monomer or 80 nM tBid. The emission maximum was 497 nm. Some batch to batch variation in absolute values was observed; however, the relative orders of binding under different conditions were retained in all cases.

Preparation of Large Unilamellar Vesicles Using an Inverted Emulsion. A previously described method (*31*) was modified to study the transbilayer diffusion of 1-hexadecanoyl-2-(1′-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (py-16-PC). Unlike the py-12-PC, this probe cannot readily be inserted from the aqueous phase into preformed liposomes because of the longer acyl chains. It is however possible to measure transbilayer diffusion with this lipid by incorporating it into one of the two monolayers of asymmetric liposomes. The asymmetric liposomes were prepared with some modifications of the method described by Pautot et al. (*31*). Two separate lipid films, with one containing py-16-PC, were deposited on the walls of glass test tubes. The lipid was suspended in dodecane by sonication under argon gas. A 2 mL sample of the homogeneous dispersion of lipid in dodecane was layered on top of 3 mL of buffer (10 mM Hepes, 0.14 M NaCl, 0.1 mM EDTA, pH 7.4) in a Falcon tube and kept at room temperature for 1−3 h to allow the lipid to diffuse to the interface. To the second lipid film was added a volume fraction of 0.5% aqueous buffer, and an emulsion was formed by vigorous vortexing and shaking. The lipid emulsion was gently layered on top of the buffer and dodecane suspension to cover the interface in the Falcon tube. The entire assembly was placed in a centrifuge and spun at 120*g* for 10 min. The liposomes formed in the lower aqueous phase were characterized using quasi-elastic light scattering as well as by fluorescence measurements. The size distribution varied somewhat among preparations with a mean diameter between 300 nm and 1.5 μm, with some heterogeneity of size for each preparation. The size distribution of a representative preparation is shown in Figure 1.

Quasi-Elastic Light Scattering (QUELS). The size distributions of the LUVs were determined with quasi-elastic light scattering using a Brookhaven model B1 9000AT digital correlator equipped with a BI-200sm goniometer, version 2.0, and a BI-900AT digital correlator system. The scattering from the sample was measured at 90° over a period of 3−5 min. The size distribution was calculated with a nonnegatively constrained least-squares algorithm, with software provided by the instrument manufacturer.

Rate of Lipid Transbilayer Diffusion Using Asymmetric Vesicles. We measured the rate of transbilayer lipid diffusion of py-16-PC by the spectrofluorometric method described above for extruded vesicles. In this case the lipid probe is incorporated into one or both of the monolayers during formation of the liposome. An aliquot of the liposomes was added to buffer in a quartz cuvette in the spectrofluorimeter. The ratio of excimer fluorescence at 476 nm to monomer emission at 376 nm was monitored using an excitation wavelength of 344 nm and 4 nm bandwidths. After 50−100 s an aliquot of a solution containing BaxΔC was added to give a final protein concentration of 600 nM. The emission ratio was recorded over a period of 20 min. Two independent preparations were made for each lipid composition. Controls without the addition of protein showed a slow change in fluorescence emission intensity, indicating a slower basal rate of flip-flop. This may be caused by the presence of a small amount of contaminating dodecane. A positive control with melittin was also run. The I_e/I_m ratios were used to calculate the transbilayer distribution of probe as briefly described above for the extruded vesicles.

Leakage Assays with Liposomes Entrapping 70 kDa Dextrans, by Antibody Quenching. A previous procedure (*32*) was modified for this assay. Lipid films with a composition of 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS, and 12 mol % CL containing 0.02 mol % N-Rh-PE were hydrated with a solution of Oregon Green−dextran (70 kDa) conjugate in PBS buffer at pH 7.4. The suspensions were vortexed and frozen−thawed five times and 100 nm diameter LUVs made by extrusion. The liposomes were passed through a 1 × 40 cm Sepharose 4B-CL gel filtration column, eluted with PBS buffer at pH 7.4 that had been adjusted to be equiosmolar. The liposomes were collected from the column in the void volume. The maximum absorbance of N-Rh-PE was used for quantifying the liposomes. Fluorescence measurements were carried out at 37 °C using an SLM Aminco Bowman Series II spectrofluorimeter, in siliconized round glass cuvettes containing 2 mL of buffer at pH 7.4, with magnetic stirring. Excitation was 488 nm and emission 528 nm, with 8 nm band-pass. LUVs were added to the buffer, and after a baseline was established BaxΔC was added. The fluorescence was followed for 20 s, and then an aliquot of a solution of fresh antibody was added to quench the fluorescence of the probe that had leaked out of the liposome. At the end of the experiment an aliquot of 20% lubrol (0.1% final concentration) was added to obtain 100% dextran release.

RESULTS

Transbilayer Diffusion of Lipids Using Extruded Liposomes. We measured the lipid dependence of transbilayer diffusion rates promoted by some Bcl-2 proteins. BaxΔC is the proapoptotic full-length Bax missing 20 amino acids on the carboxyl terminus. Unlike the full-length Bax, it remains oligomeric in the absence of detergent. In all cases, the movement of the py-12-PC was used as the probe. For both BaxΔC (Figure 2) and another proapoptotic Bcl-2 protein, tBid (Figure 3) CL promotes greater rates of lipid transbilayer diffusion than does DOPG. In addition, with liposomes of the same lipid composition, BaxΔC promotes more membrane permeabilization than tBid. This is consistent with the relative leakage potency of BaxΔC, full-length Bax (*11*), or

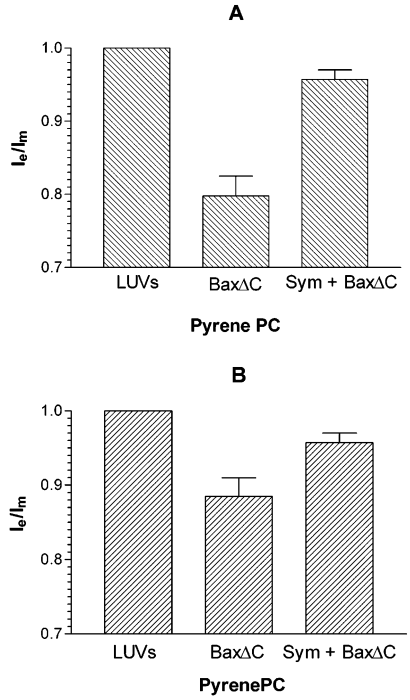

FIGURE 2: Rate of transbilayer diffusion of py-12-PC. A decrease in the ratio of I_e (intensity of excimer emission) to I_m (intensity of monomer emission) reflects transbilayer diffusion of py-12-PC. The ratio I_e/I_m has been normalized to a value of 1 with LUVs alone. The extent of this translocation is measured over 200 s and is negligible for LUVs in the absence of protein (first bar). Liposomes were at 20 μM and were composed of DOPC/CL (2:1) (A) or 10 μM DOPC/DOPG (7:3) (B). The BaxΔC is present at 100 nM. Symmetrically labeled control liposomes (labeled Sym + BaxΔC) were also treated with 100 nM BaxΔC. The error bars correspond to standard deviations of measurements carried out in two different batches of liposomes.

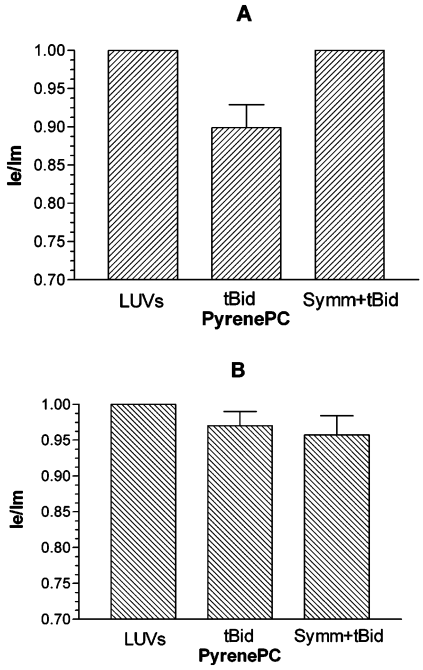

FIGURE 3: Rate of transbilayer diffusion of py-12-PC. The ratio I_e/I_m has been normalized to 1 with LUVs alone. The extent of this translocation is measured over 200 s and is negligible for LUVs in the absence of protein (first bar). Liposomes were at 20 μM and were composed of DOPC/CL (2:1) (A) or 10 μM DOPC/DOPG (7:3) (B). The tBid is present at 80 nM. Symmetrically labeled control liposomes (labeled Symm) were also treated with 80 nM tBid. The error bars correspond to standard deviations of measurements carried out in two different batches of liposomes.

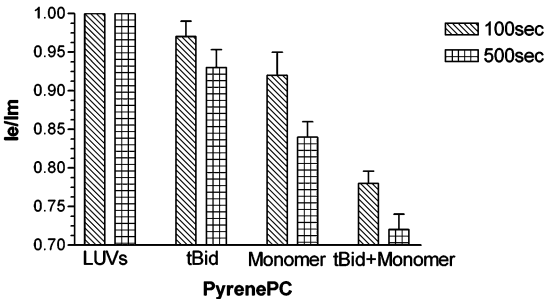

FIGURE 4: Rate of transbilayer diffusion of py-12-PC. The extent of this translocation is measured over 100 and 500 s and is shown by pairs of bars. The rate is negligible for LUVs in the absence of protein (first pair of bars). Liposomes were at 10 μM and were composed of 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS, and 12 mol % CL to mimick the composition of the outer membrane of mitochondria. The monomeric full-length Bax (monomer) is present at 100 nM and the tBid at 80 nM. The error bars correspond to standard deviations of measurements carried out in two different batches of liposomes.

tBid (*34*). The results also demonstrate that the ability of these proteins to promote flip-flop is not strictly dependent on the presence of a particular lipid. This was also confirmed with measurements of the flip-flop rate of py-12-PS using liposomes of varying lipid composition (not shown). The rate of flip-flop of py-12-PS was similar to but somewhat slower than that of py-12-PC. The slower rate of the PS probe is likely caused by the more polar headgroup. It is known that the rate of transbilayer diffusion is dependent on the structure of the lipid (*35*−*37*). Thus, these proteins likely promote transbilayer diffusion by a mechanism that is not specific to a particular lipid structure.

The monomeric form of full-length Bax weakly increases the rate of lipid transbilayer diffusion in liposomes composed of a lipid mixture mimicking the lipid composition of the mitochondrial outer membrane (*12*) (41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS, and 12 mol % CL) (Figure 4). The protein tBid has almost no effect on this rate. However, tBid combined with the monomeric form of full-length Bax significantly enhances the rate of lipid transbilayer diffusion (Figure 4). The rate of flip-flop of pyr-12-PC is very slow in the absence of protein (LUVs). A control showed an about 5% decrease in I_e/I_m when BaxΔC was added to liposomes containing symmetrically distributed probe (not shown). This confirms that the decrease in excimer/monomer ratio is caused by lipid dilution due to flip-flop. The I_e/I_m ratios were converted to q, defined as the fraction of labeled lipid that has translocated to the inner monolayer (Figure 5). The kinetics does not correspond to a simple first-order rate process but exhibits a more rapid initial phase. It is possible that the more rapid phase is driven by a mass imbalance between the inner and outer monolayers after the external addition of protein (*33*). This type of kinetic behavior is similar to that previously reported for melittin and magainin, using the present method of measurement (*24*).

The rate of transbilayer diffusion with BaxΔC, full-length monomeric Bax, or tBid correlated well with the membrane binding of these proteins to liposomes (Figure 6). Neither the monomeric form of Bax nor tBid binds to the membrane, but if these two proteins are premixed, they can translocate to the membrane, where they promote transbilayer diffusion.

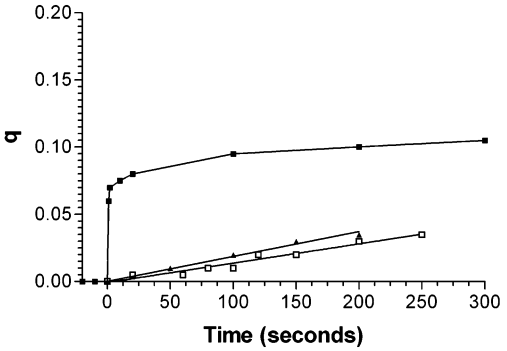

FIGURE 5: Time dependence of the fraction of py-12-PC that has undergone transbilayer diffusion (*q*) in the presence of 80 nM tBid (▲), 10 nM monomeric full-length Bax (□), or a premixed solution giving a final concentration of 80 nM tBid and 10 nM monomeric full-length Bax (■).

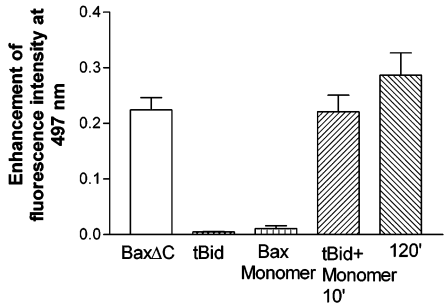

FIGURE 6: Binding of (1) BaxΔC (100 nM), (2) tBid (80 nM), (3) monomeric full-length Bax (100 nM), and (4, 5) a premixed combination of tBid and monomeric full-length Bax. Liposomes were at 10 μM and composed of 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS, and 12 mol %. A Ca^{2+}−EGTA buffer in 10 mM Hepes, 0.14 M NaCl, pH 7.4, containing 100 nM free Ca^{2+} was used. A 1 mM concentration of Mg^{2+} was added to the LUVs and proteins. Binding in first four bars was measured after 10 min; in bar 5 it was measured after 120 min. The error bars are standard deviations of three independent measurements carried out with one batch of liposomes.

Transbilayer Diffusion of Lipids Using Asymmetric Liposomes. An important property of biological membranes, that of their transbilayer asymmetry, is mimicked in asymmetric liposomes. Preparing liposomes in this manner also allows the use of a pyrene-labeled lipid with longer acyl chains, typical of acyl chain lengths found in lipids from biological sources. We measured the BaxΔC-induced increase in transbilayer diffusion rates of py-16-PC using liposomes made with an outer monolayer containing POPC and 10 mol % py-16-PC and an inner monolayer containing POPC, POPE, and CL (1:1:1) (Figure 7, curve 2) or with an outer monolayer containing POPC, POPE, and CL (1:1:1) and an inner monolayer containing POPC and 10 mol % py-16-PC (Figure 7, curve 3). For both liposomes there is a significant increase in the rate of transbilayer diffusion of lipids compared to the case in which BaxΔC was not added (Figure 7, curve 1). Although still slow, there is a higher rate of spontaneous flip-flop in these liposomes compared with the extruded ones possibly because of the presence of contaminating dodecane. The spectrum of the probe, corresponding to curve 2, is shown as an example (Figure 8). Immediately after the addition of BaxΔC there is little change in the spectral shape. Although only the control curve 1 is shown (Figure 7), with liposomes having the same lipid composition as for curve 2 (Figure 7), controls with liposomes corresponding to those used for curve 3 (Figure 7), but without Bax, were similar (not shown). After addition of BaxΔC, the liposomes having CL in the outer monolayer (curve 3, Figure 7) have a somewhat higher rate of flip-flop, likely because of increased binding of BaxΔC. Converting the I_e/I_m ratio to the fraction of probe that has undergone transbilayer diffusion (*q*) demonstrates that the rate is biphasic, as shown with the LUVs (Figure 9).

To test whether the increased rate of transbilayer diffusion is accompanied by the formation of a large pore, we

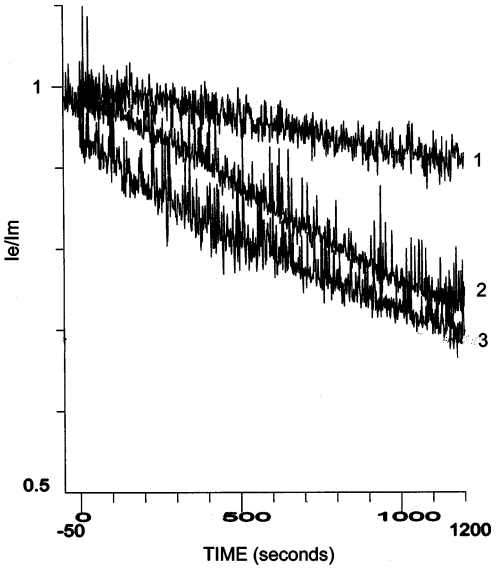

FIGURE 7: Time course of transbilayer diffusion of py-16-PC in asymmetric liposomes. Curve 1: control with no protein addition. Liposomes have an outer monolayer containing POPC and 10 mol % py-16-PC and an inner monolayer containing POPC, POPE, and CL (1:1:1). Curve 2: same liposomes as for curve one but with the addition of 600 nM BaxΔC. Curve 3: liposomes with an outer monolayer containing POPC, POPE, and CL (1:1:1) and an inner monolayer containing POPC and 10 mol % py-16-PC with the addition of 600 nM BaxΔC.

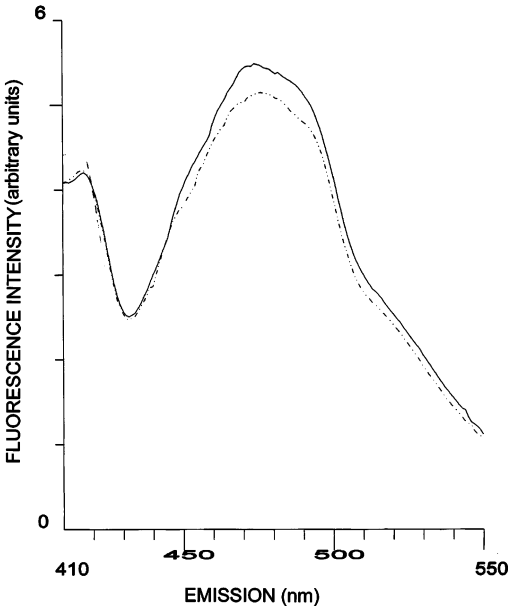

FIGURE 8: Emission spectra of asymmetric liposomes with an outer monolayer containing POPC and 10 mol % py-16-PC and an inner monolayer containing POPC, POPE, and CL (1:1:1). Solid curve: prior to the addition of 600 nM BaxΔC. Dashed curve: shortly after the addition of 600 nM BaxΔC.

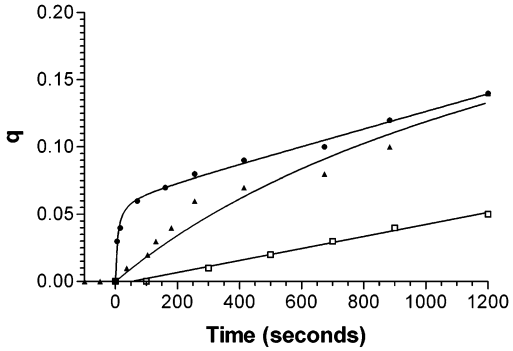

FIGURE 9: Time dependence of the fraction of py-12-PC that has undergone transbilayer diffusion (*q*) in the presence of 600 nM BaxΔC. Curves correspond to those of Figure 7: curve 1 (□); curve 2 (▲); curve 3 (●).

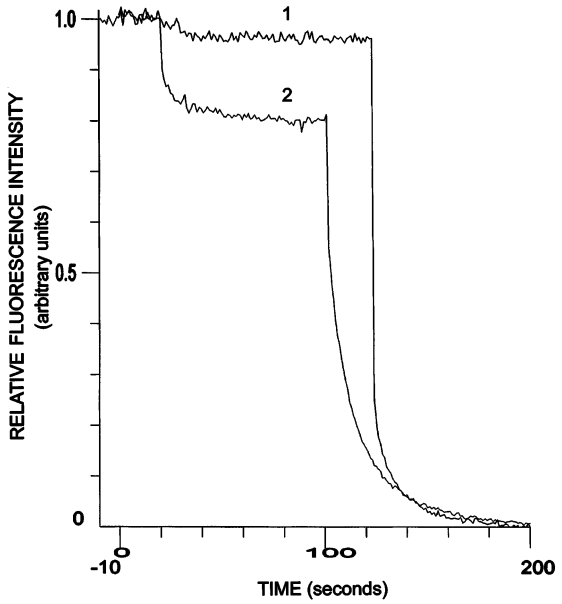

FIGURE 10: BaxΔC can release 70 kDa dextran from 50 uM LUVs with the composition simulating the outer mitochondrial membrane. The experiment was done at 37 °C. Curve 1: antibody control added at 20 s to LUVs. Curve 2: antibody added at 20 s to LUVs containing 100 nM BaxΔC. Fluorescence emission was followed with time at 528 nm.

measured the permeabilization of a 70 kDa labeled dextran (Figure 10). With 100 nM BaxΔC this entrapped molecule did undergo partial release, indicating that the release of entrapped material was through a pore larger than 35 nm in diameter. This partial release occurs together with the observed increased rate of phospholipid transbilayer diffusion.

DISCUSSION

An aspect of biological membranes that has rarely been incorporated into model systems is that of the transbilayer asymmetry of lipids. We apply a modification of a recently developed method for preparing asymmetric liposomes (*31*) and demonstrate that they are suitable for measuring rates of transbilayer diffusion of lipids. In addition, we also use a method that allows the asymmetric incorporation of labeled lipid in the liposome to measure lipid transbilayer diffusion. Unlike methods involving quenching of probes in the outer monolayer with small molecules, the two methods we have used can measure rates of transbilayer diffusion independent of the presence of pores in the membrane. This is important in the present case since we can directly demonstrate that BaxΔC causes the formation of pores large enough to release 70 kDa dextran. This is consistent with images from atomic force microscopy indicating the formation of large pores by full-length oligomeric Bax in membrane bilayers (*38*).

The process of transbilayer lipid diffusion may also be important for the action of Bax on mitochondria. Cardiolipin has been suggested to play an important role in apoptosis through the release of cytochrome *c* into the cytosol (*39*, *40*). It has been shown that liposomes containing cardiolipin are more prone to leakage in the presence of Bax than those containing the structurally related lipid phosphatidylglycerol (*11*, *12*). Cardiolipin has also been suggested to play an important role in the action of Bax in mitochondria (*10*). However, it is also known that cardiolipin is lost during apoptosis (*41*−*43*). This apparent anomaly can be explained by the fact that the loss of cardiolipin is a later event in apoptosis that facilitates the release of cytochrome *c* from a membrane-bound state (*42*). Initially, the presence of cardiolipin likely enhances the action of the Bax. However, it has recently been shown in yeast mitochondria that CL is not required for Bax-mediated cytochrome *c* release (*44*).

In intact mitochondria, the major fraction of cardiolipin is on the inner mitochondrial membrane (*28*), while Bcl-2 proteins initially contact the outer leaflet of the outer mitochondrial membrane (*30*). Furthermore, there is evidence that cardiolipin is present on the inner leaflet of the inner membrane of the mitochondria (*14*). For cardiolipin to become exposed on the surface of mitochondria, in addition to undergoing flip-flop, it also must either exchange between the inner and outer membrane bilayer of the mitochondria or enter into "contact sites", at which the inner and outer mitochondrial membranes join. There is evidence that these contact sites are enriched in cardiolipin (*17*, *18*). Thus, initially, prior to apoptosis, there is a small amount of cardiolipin exposed on the surface of mitochondria. In the presence of Bcl-2 proteins, after initiation of apoptosis, larger amounts of cardiolipin can be transferred from the inner to the outer mitochondrial membrane (*19*, *20*). Although there are pathways for cardiolipin to become exposed to the outer surface of mitochondria in resting cells, the net consequence of cardiolipin movements is to maintain a high degree of lipid asymmetry, with most of the lipid being sequestered to the inner leaflet of the inner mitochondrial membrane. It is also known that upon initiation of apoptosis there is a net movement of cardiolipin to the surface of mitochondria. There may be several pathways by which this occurs, but the present work indicates that Bax can contribute directly to this process by accelerating the rate of lipid transbilayer diffusion.

The action of Bax in promoting flip-flop is dependent on the protein being in an active, oligomeric state. Thus, the monomeric full-length Bax cannot promote flip-flop nor the formation of pores in membranes. However, BaxΔC that remains oligomeric even after removal of detergent can promote both flip-flop and membrane leakage. The pore that is formed with this protein is large, and the concomitant promotion of lipid flip-flop indicates that it is lined with both lipid and protein. The presence of lipid and protein in the pore formed with Bax has been previously suggested, on the basis of other evidence (*22*, *45*).

There is evidence for increased rates of cardiolipin transfer between mitochondrial membranes with both Bid and tBid (*46*). However, for this lipid to appear at the outer surface of the mitochondria, it must also undergo transbilayer diffusion. We find little effect of tBid in promoting this process. However, tBid is capable of strongly enhancing the action of full-length monomeric Bax in promoting flip-flop. This is accompanied by an increased binding of these proteins to membranes and by an increase in the rate of leakage across the membrane (*11*). The increased rate of lipid transbilayer diffusion caused by activated Bax combined with lipid transfer induced by tBid would explain how cardiolipin can become exposed to the cytosol.

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