tBid forms a pore in the liposome membrane

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Received 20 October 2003; revised 10 November 2003; accepted 12 November 2003

First published online 25 November 2003

Edited by Vladimir Skulachev

Abstract **We investigated the ability of tBid (truncated form of Bid) to bind and permeabilize the liposomes (large unilamellar vesicles, LUVs) and release fluorescent marker molecules (fluorescein-isothiocyanate-conjugated dextrans, FITC-dextrans) of various molecular diameters (FD-20, FD-70, FD-250S) from LUVs. Obtained data showed that tBid was more efficient in promoting leakage of FITC-dextrans from LUVs composed of cardiolipin and dioleoylphosphatidylcholine (DOPC) than LUVs made of dioleoylphosphatidic acid or dioleoylphosphatidylglycerol and DOPC. The leakage efficiency was reduced with increasing amount of dioleoylphosphatidylethanolamine or dielaidoylphosphatidylethanolamine. Phospholipid monolayer assay and fluorescence quenching measurements revealed that tBid inserted deeply into the hydrophobic acyl chain of acidic phospholipids. Taking into account the tBid three-dimensional structure, we propose that tBid could penetrate into the hydrophobic core of membrane, resulting in the leakage of entrapped content from LUVs via a pore-forming mechanism.**

Key words: tBid; Large unilamellar vesicle; Leakage; Pore formation mechanism

1. Introduction

Mitochondria are viewed as one of the most pivotal sensors and amplifiers in an apoptotic process by releasing apoptogenic proteins including cytochrome *c*, AIF (apoptosis-inducing factor), Smac/DIABLO and endonuclease G [1,2]. It has been proven that the release of these proteins is mainly regulated by Bcl-2 protein family members, which constitute a critical checkpoint in the apoptotic cascade [3,4]. Members of this family include both pro- and anti-apoptotic molecules, which are characterized by the presence of up to four conserved regions termed Bcl-2 homology (BH) domains (BH1–BH4). Bid is an abundant proapoptotic protein of the Bcl-2 family that shares its sequence homology only within the BH3 amphipathic α-helical region. Emerging evidence indicates that Bid is involved in various pathways of apoptosis that interplay the activation of caspases with mitochondrial dysfunction [5,6]. Full-length Bid is usually localized in the cytosolic fraction of living cells and activated through proteolytic cleavage by caspase 8 following treatment with TNFα or anti-Fas. After the cleavage, the truncated product, tBid, translocates to the mitochondria and facilitates the release of cytochrome *c*, as well as other proteins resident in the intermembrane space.

tBid might serve as a death ligand to induce the release of cytochrome *c* from mitochondria by promoting conformational change of other proteins, such as Bax or Bak [7,8]. Alternatively, it is also conceivable that tBid alone may be capable of destabilizing lipid membranes in vitro [9,10]. Studies using model membrane systems reveal that tBid can form ion channels in planar bilayers [11] and thus lead to the leakage of the entrapped small molecules, such as 5,6-carboxyfluorescein or ANTS/DPX, as well as large molecules such as cytochrome *c* or trypsin, from phospholipid vesicles [9,10,12,13]. These results propose that Bid can function on its own as a downstream effector directly on the mitochondrial outer membrane. Of note, studies with recombinant Bid indicate that protein–lipid interactions leading to membrane permeation play a major role on its activity. It has been reported that tBid may specifically interact with cardiolipin, a negatively charged lipid characteristically located on the inner mitochondrial membrane as well as in the inner–outer membrane contact sites [14]. Moreover, recent evidence suggests that Bid has an intrinsic capacity of binding and exchanging membrane lipids, which is similar to plant lipid transfer proteins [15,16]. This unique similarity of Bid with unrelated proteins suggests common interactions with lipids. The biochemical mechanism by which Bid performs its function on mitochondria remains uncertain. The three-dimensional structure of Bid shows a bundle of eight α-helices with two central predominantly hydrophobic helices forming the core of the molecule [17,18], which is reminiscent of pore-forming regions in the bacterial toxins of diphtheria toxin fragment B and colicin. Recently, recombinant tBid has been reported to form oligomers in the presence of non-ionic detergents or mitochondrial lipids [19]. These data lend credence to the hypothesis that tBid oligomers may form pores in the mitochondrial membranes.

Many studies have revealed that membrane permeation by amphipathic α-helical peptides occurs via either a pore-forming barrel stave mechanism or a non-pore carpet-like mechanism [20]. By using model membrane systems to determine the possible mechanism that tBid involves in, we detected in detail the abilities of tBid to interact with phospholipid membranes and to induce the leakage of fluorescein-isothiocyanate-con-

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Abbreviations: tBid, truncated form of Bid; LUVs, large unilamellar vesicles; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; CL, 1,1′,2,2′-tetraoleoyl cardiolipin; 12-DPC, 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine; FITC-dextran, fluorescein-isothiocyanate-conjugated dextran; PM, *N*-(1-pyrenyl) maleimide

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01346-2

jugated dextrans (FITC-dextrans) with various molecular diameters from large unilamellar vesicles (LUVs) of different phospholipid compositions. In light of these results, we tentatively postulated a pore-forming mechanism by which tBid permeates the phospholipid membranes and induces the leakage of encapsulated content from LUVs. This may represent an alternative mechanism of tBid for inducing mitochondrial dysfunction and apoptosis.

2. Materials and methods

2.1. Materials

All chemical reagents were of the highest purity available commercially. 1,2-Dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE), 1,1′,2,2′-tetraoleoyl cardiolipin (CL) and 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine (12-DPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). HEPES, lubrol PX, FITC-dextrans: FD-20 (molecular weight (MW) = 21 200 Da), FD-70 (MW = 71 600 Da), FD-250S (MW = 282 000 Da) were products of Sigma (St. Louis, MO, USA). *N*-(1-pyrenyl) maleimide (PM) was obtained from Molecular Probes (Junction City, OR, USA).

2.2. Expression and purification of recombinant proteins

Murine Bid expression plasmid was kindly provided by Dr. Bruno Antonsson, Serono Pharmaceutical Research Institute, Switzerland. Caspase 8 expression plasmid was kindly provided by Dr. Xiaodong Wang, Southwestern Medical Center in Dallas, TX, USA. The plasmids for expression of Bid and caspase 8 were separately transformed into bacterial BL21(DE3) cells. Then the recombinant proteins were purified from the cell lysate using nickel affinity column (Qiagen, USA), and Bio-scale Q5 column (Bio-Rad, USA) was used for further purification. tBid was obtained by adding 1/50 (v/v) caspase 8 to the full-length Bid, then incubating overnight at 4°C. The cleavage product containing N- and C-terminal fragments can not be separated because the dissociation normally requires micellar concentrations of non-ionic detergents. To avoid the possible effect of detergent, the mixture of the two fragments was used for assays since it has comparable activity to the isolated C-terminal fragment [9].

2.3. Preparation of liposomes

LUVs encapsulated with different FITC-dextrans (FD-20, FD-70, FD-250S) were prepared. 2.5 ml diethyl ether and 0.5 ml HEPES buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA)) containing FITC-dextrans (5 mg/ml) were added to a dry lipid film of 5 μmol indicated phospholipids. After sonication for 20 min at 4°C with a bath sonicator, and evaporation of ether under reduced pressure, the vesicles were dialyzed overnight at 4°C in HEPES buffer. Small unilamellar vesicles (SUVs) were prepared by sonicating in HEPES buffer on a sonicator at 4°C under nitrogen purge. After sonication, the solution was centrifuged at 100 000 × *g* for 30 min on a Beckman TL-100 ultracentrifuge to eliminate the multilayer liposomes.

The phospholipid concentration was determined by perchloric acid digestion [21].

2.4. Assay for leakage of liposomal contents

The leakage assay was performed as the methods described in [22]. LUVs containing FITC-dextrans were prepared by reverse-phase evaporation, and separated from extravesicular FITC-dextrans by washing. Following incubation of increasing amounts of tBid with 100 μM LUVs in 100 μl of HEPES buffer at 30°C for 30 min, the vesicles were sedimented by centrifugation (25 min, 35 000 rpm, 4°C). Half of the supernatant (S) was sampled out, to which, and to the remainder (R) in the centrifugation tube as well, 350 μl HEPES buffer containing 0.1% lubrol PX was added to dilute and dissolve the samples. The control followed the same procedure except for the addition of buffer instead of protein. FITC-dextrans contained in the two parts of the solution were quantified using a Hitachi F-4010 spectrofluorometer, and the fluorescence intensity corrected for self-quenching according to the standard curve of fluorescence versus FITC-dextrans. FITC-dextrans were excited at 497 nm and emission at 520 nm was recorded with bandwiths of 5 nm for both excitation and emission. The percentage of leakage, therefore, was determined by

$$\text{leakage}\ (\%) = \left(\frac{2\times S}{S+R} - B\right)\times 100$$

where B is the leakage extent of the control.

2.5. Insertion of tBid into phospholipid monolayer

A film balance, type Han-2000, designed and made in our laboratory was used to study membrane insertion ability of proteins. Briefly, 3 ml HEPES buffer was added into the mini-trough as a subphase followed by dropping the phospholipids on the surface of buffer to form monomolecular lipid layer, which gave the desired initial surface pressure. After the initial surface pressure stabilized to a plateau value, the appropriate amount of protein was injected to the mixing chamber with a magnetic stir bar through a 0.7 cm^2 hole in the edge. Then the samples rapidly diffused to the monolayer-spreading disk to make an increase of the surface pressure. All measurements were performed at room temperature.

2.6. Fluorescence measurement

Fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer at 30°C. PM, that normally modifies cysteine residues, was employed to derivatize tBid. Briefly, tBid was dialyzed exhaustively against Tris buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl) before chemical modification. Protein was modified at a molar ratio of 3 mol of PM to 1 mol tBid for 2 h at 30°C. After the reaction, the protein sample was dialyzed against Tris buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl). The fluorescence intensity of PM-labeled tBid with indicated liposomes was measured with excitation and emission wavelength at 340 and 376 nm, respectively. Slits of 2.5 nm were used.

Fluorescence quenching was carried out with nitroxide spin-labeled 12-doxyl phosphatidylcholine (12-DPC). 12-DPC was incorporated into the SUVs at a concentration of 10% (molar percent). Aliquots of SUVs with or without 12-DPC were added to the cuvette containing PM-labeled tBid (1 μM). Fluorescence intensities in the absence (F_0) and the presence (F) of 12-DPC were corrected by subtracting intensities measured without protein. The F_0/F values were obtained from the corrected spectra.

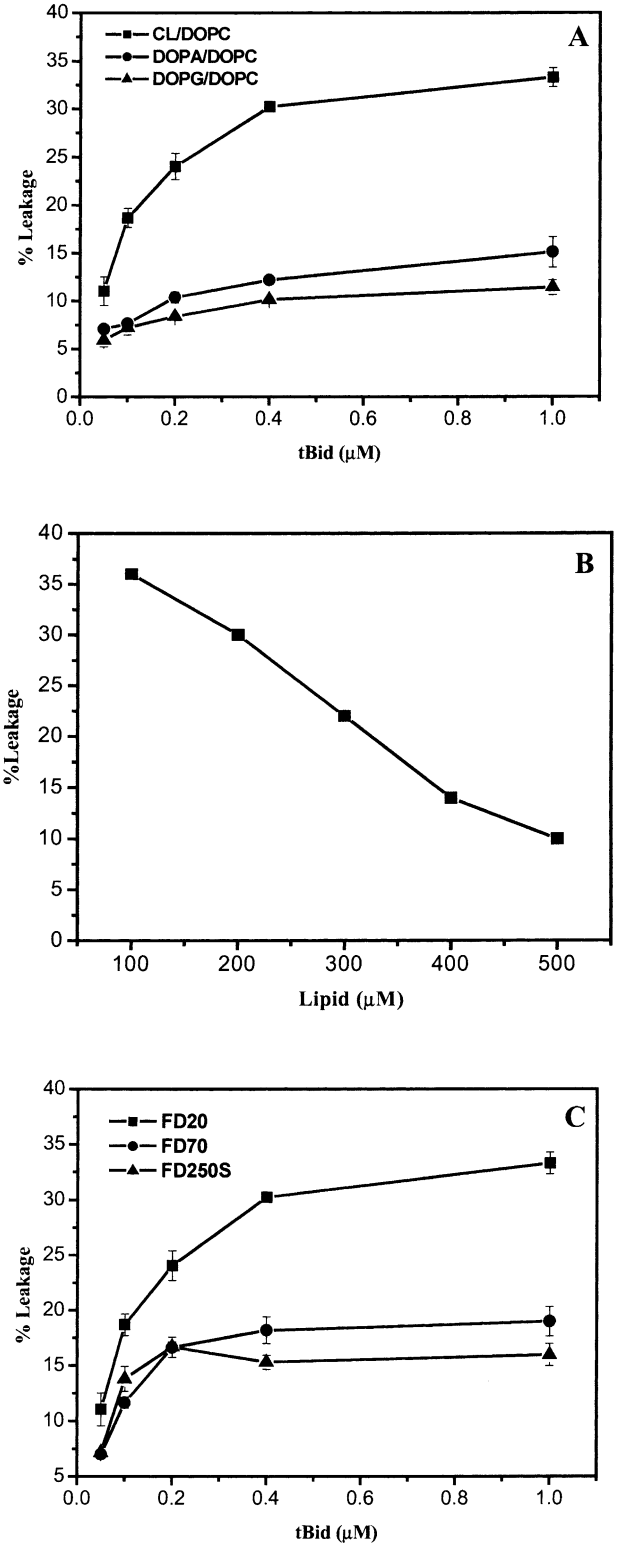
3. Results

3.1. Leakage of FITC-dextrans from LUVs

It has been reported that cardiolipin provides specificity for targeting tBid to mitochondria and then efficiently facilitates the release of cytochrome *c* [14]. Herein, tBid-induced leakage of FITC-dextran (FD-20) from LUVs composed of CL/DOPC, DOPA/DOPC or DOPG/DOPC, at ratios 1:2 (mol:mol), was studied and compared. As shown in Fig. 1A, tBid induced the leakage to a larger extent in LUVs containing cardiolipin than in those containing DOPA and DOPG. Thus, cardiolipin is important not merely for the membrane targeting of tBid, but also for its permeabilization function. Also, leakage has been studied as a function of constant tBid concentration and lipid concentration with LUVs composed of CL/DOPC (1:2 molar ratio). When lipid concentration was held constant, increasing amount of tBid induced a greater extent of leakage until a plateau value was reached (Fig. 1A). With constant tBid concentration at 1 μM, increasing the lipid concentration led to a decrease of leakage (Fig. 1B). Since increasing the lipid concentration increased the number of vesicles in the suspension, the results were consistent with the hypothesis that a defined number of proteins is required for the pore formation in bilayer.

To clarify the possible mechanism by which tBid permeates the liposomes, we studied the leakage induced by tBid from

the LUVs encapsulated with FITC-dextrans of different molecular weights. The molecular diameters of three FITC-dextrans, FD-20 (MW = 21 200 Da), FD-70 (MW = 71 600 Da) and FD-250S (MW = 282 000 Da), were measured by Dynao-Pro LSR-TC. The measurements showed that the molecular diameters increased correspondingly with the molecular weights of FITC-dextrans. The tBid-induced leakage of FD-20, FD-70 and FD-250S separately encapsulated in LUVs composed of cardiolipin and DOPC (with molar ratio 1:2) was assayed. The results revealed that the leakage extent of FD-20 was higher than that of either FD-70 or FD-250S (Fig. 1C). These results suggest that the mechanism of the formation of discrete pores might be involved in the leakage process as well, since the leakage efficiency of markers with different molecular weights would be the same if these results were due to the carpet-like mechanism [20].



3.2. The effect of phosphatidylethanolamine (PE) on the leakage

PE is abundant on the outer mitochondrial membrane. To investigate if PE will affect the tBid-induced leakage, the efficiency of leakage of FD-20 from LUVs made of CL/DOPC (1:2 molar ratio) and CL/DOPC/DOPE (1:1:1 molar ratio) were compared. Notably, the presence of DOPE decreased the leakage of FD-20 from LUVs (Fig. 2A). CL and DOPE are both known as non-lamellar phase phospholipids. To exclude the possible effect of non-bilayer-forming tendency of CL, liposomes containing DOPG combined with different percentages of DOPE were used in the further leakage assays. As shown in Fig. 2B, DOPE caused a clear drop of tBid-induced leakage of FD-20 from DOPG-containing LUVs, and with its content increasing, the leakage efficiency was gradually reduced. Furthermore, DEPE, without the non-bilayer-forming tendency, was tested (Fig. 2C). Very interestingly, similar to DOPE, the presence of DEPE lowered FD-20 leakage from the DOPG-containing LUVs. Higher proportions of DEPE led to lower leakage efficiency. These results resembled those obtained from the pore-forming peptides such as GALA or magainin-2, which were similarly affected by DOPE [23,24].

3.3. The insertion ability of tBid into phospholipid monolayer

An increase in the surface pressure is observed when a protein is inserted into the phospholipid monolayer. Usually, an increase of the surface pressure $\Delta\pi$ is measured as a function of the initial surface pressure (π). A plot of $\Delta\pi$ versus π yields a straight line with a negative slope that intersects the abscissa at the value named as the limiting surface pressure π_c. The higher π_c value means stronger penetration ability of protein. In monolayer, the 'equivalence pressure' of the bilayer may be defined as the pressure at which the lipid density in the monolayer is identical to that in the bilayer. The bilayer equivalence pressure of the monolayer is thought to be 32–35

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Fig. 1. tBid-induced leakage of FITC-dextrans from LUVs. tBid was incubated with indicated LUVs encapsulated with FITC-dextrans. After an incubation at 30°C for 30 min, centrifugation was carried out at 35 000 rpm for 25 min at 4°C. The FITC-dextrans release from LUVs was detected by fluorescence analysis of the supernatant and pellet from each sample using a Hitachi F-4010 spectrofluorometer. A: Increasing amount of tBid was incubated with LUVs at constant concentration 100 μM and induced the release of FD-20 from LUVs composed of CL/DOPC, DOPA/DOPC or DOPG/DOPC, at ratios 1:2 (mol:mol). B: Effect of lipid concentration on the leakage efficiency of LUVs was assayed. The tBid concentration was kept constant at 1 μM while the concentration of LUVs composed of CL/DOPC (1:2 molar ratio) was varied to obtain the desired ratio. C: tBid induced the release of FITC-dextrans with various molecular diameters (FD-20, FD-70, FD-250S) from LUVs composed of CL/DOPC (1:2 molar ratio).

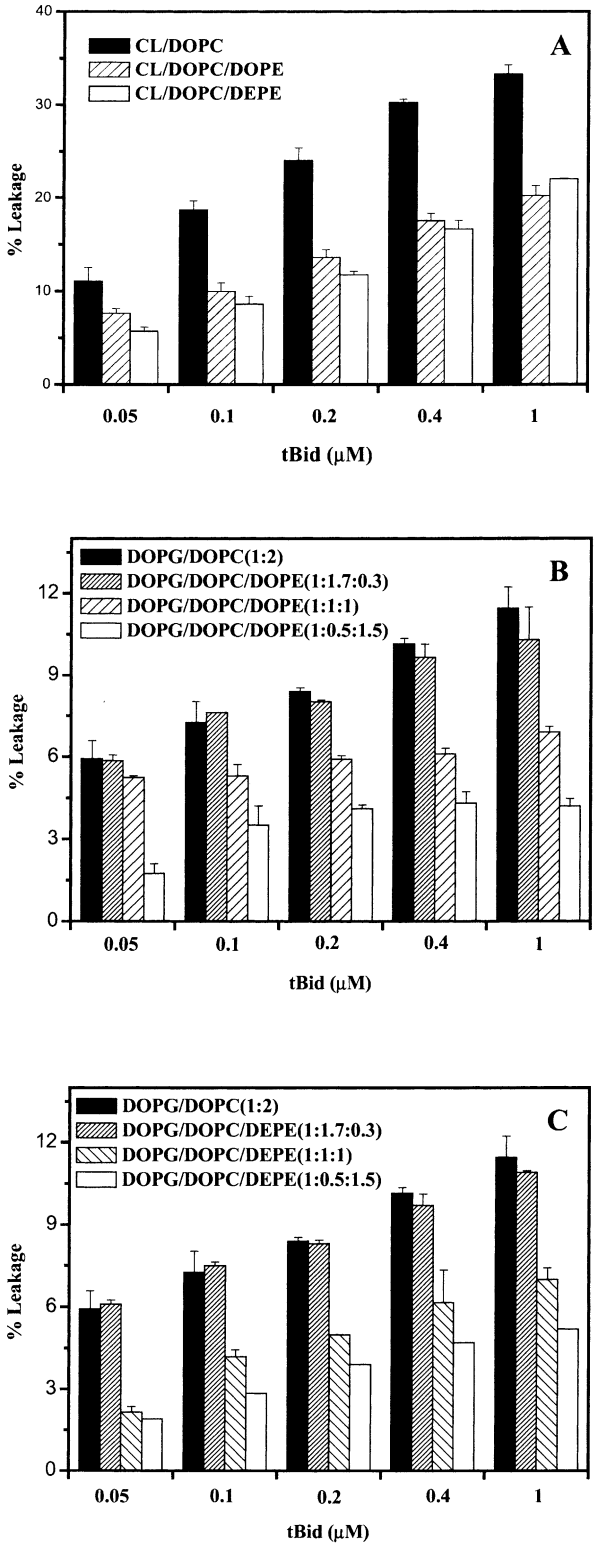


Fig. 2. The effect of PE on the leakage. A: Effect of increasing amount of tBid on the leakage of FD-20 from 100 μM LUVs of CL/DOPC (1:2 molar ratio) and CL/DOPC/DOPE (1:1:1 molar ratio). B, C: In the LUVs composed of DOPG/DOPC/DOPE or DOPG/DOPC/DEPE, the fraction of DOPG was kept constant and the content of DOPE or DEPE changed from 10, 30 to 50 mol%. The fluorescence intensity of FD-20 leaking from indicated LUVs with increasing tBid amount was detected as described in Section 2.

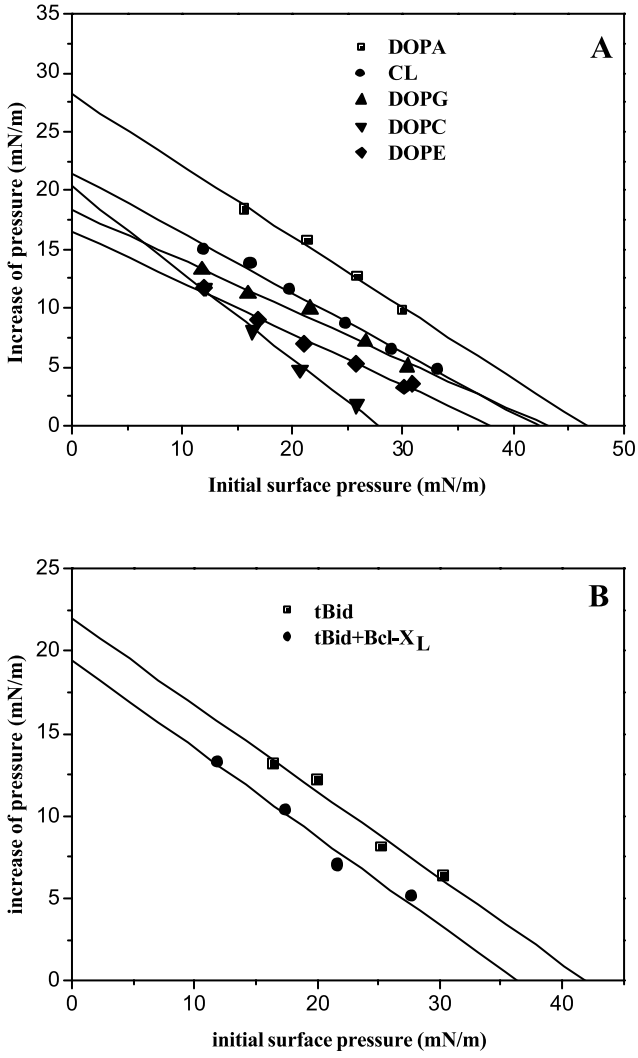
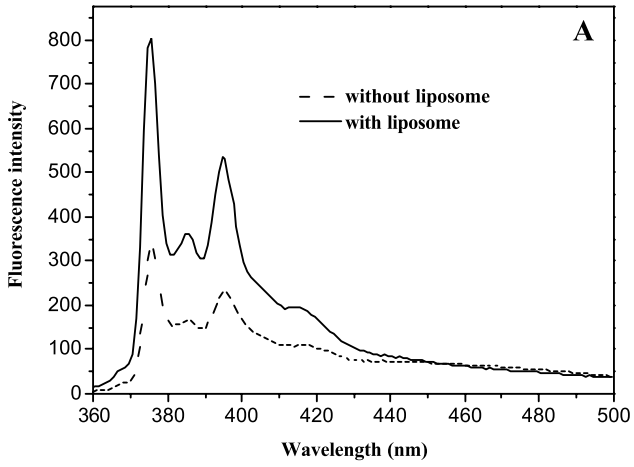
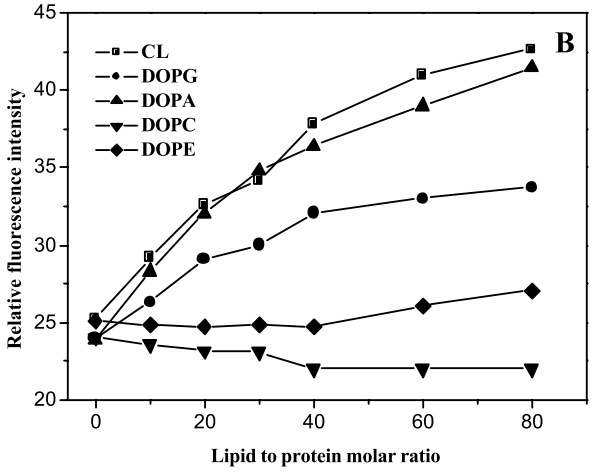


Fig. 3. Insertion of tBid into phospholipid monolayer. Relations between the initial surface pressure and the surface pressure increase were detected. A: Displayed were the π–$\Delta\pi$ plots of surface pressure changes after injection of tBid (0.5 μg/ml) underneath indicated phospholipid monolayer at different initial surface pressures. The limiting pressures of tBid in CL, DOPA, DOPG, DOPE and DOPC were 42, 45, 43, 37 and 28 mN/m, respectively. B: Bcl-X_L functioned as an inhibitor of tBid inserting into phospholipid monolayer. The limiting pressure of tBid reduced when Bcl-X_L was present.

mN/m [25], hence the π_c is a more influential factor in predicting the insertion ability of a protein into the membrane.

As shown in Fig. 3A, the penetration ability of tBid in acidic phospholipids CL, DOPA or DOPG was comparable, but was higher than in either DOPE or DOPC. The π_c of tBid in CL, DOPG and DOPA was 42, 43 and 45 mN/m, respectively, while for DOPE and DOPC, the π_c value was 37 and 28 mN/m, respectively. The results revealed that tBid has more potential to insert into acidic phospholipid membranes, but showed less insertion into neutral phospholipid DOPC or DOPE. That is, the acidic phospholipid was necessary for the insertion of tBid into lipid monolayer. Prior studies had proposed that Bcl-X_L could interact with the BH3 domain of Bid through its hydrophobic cleft to prevent the leakage induced by Bid [7]. Herein, the π_c value of tBid penetrating into phospholipid monolayer was diminished when Bcl-X_L was added





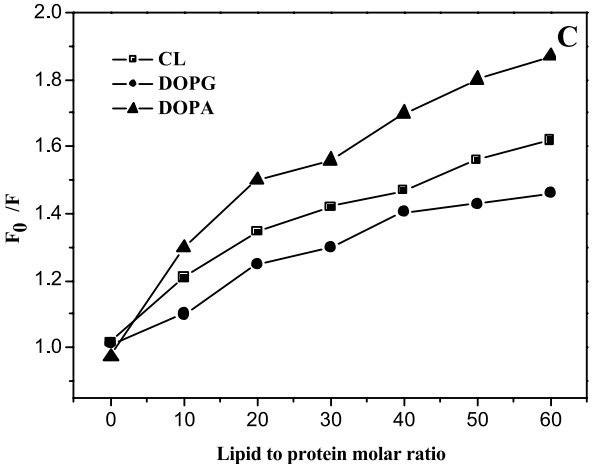


Fig. 4. Demonstration of tBid penetrating into acidic phospholipid vesicles. A: Fluorescence spectra of PM-labeled tBid before and after vesicles of CL were added. The fluorescence spectra were monitored with excitation wavelength at 340 nm and emission from 360 to 500 nm. B: The relative fluorescence intensity of PM-tBid was assayed as a function of the ratio of liposomes/PM-tBid (molar ratio) with various phospholipid vesicles. C: Quenching of PM-labeled tBid fluorescence by 12-DPC. 1 μM PM-tBid was added to indicated phospholipid vesicles with or without 12-DPC. F_0 and F represent the fluorescence intensities measured using PM-tBid/phospholipid vesicles in the absence or presence of 12-DPC, respectively.

(Fig. 3B), which indicated that the interaction of tBid with Bcl-X_L prevented its further insertion into the membrane.

3.4. Penetration of tBid into acidic phospholipid vesicles

To further study tBid insertion into phospholipid bilayers, the environmentally sensitive pyrene (PM) probe was used as a positional marker of tBid. By virtue of its chemical specificity toward sulfhydryl group [26], PM could be an ideal candidate to probe the free sulfhydryl group Cys-126 in the H5 helical region of tBid. Fig. 4A shows that the fluorescence intensity increased when CL-containing vesicles were introduced to PM-labeled tBid, which implied the penetration of the protein into the hydrophobic region of the membrane. Furthermore, changes in the fluorescence intensities of PM-labeled tBid following its interaction with various phospholipid vesicles were measured (Fig. 4B). The results revealed that, in accordance with the studies of the phospholipid monolayer, tBid could penetrate into the membrane bilayer made of CL, DOPA or DOPG, but not of DOPC or DOPE.

Moreover, the location of the PM-labeled cysteine residue with respect to the phospholipid membranes was determined by fluorescence quenching with spin-labeled derivatives of phosphatidylcholine (PC). 12-DPC contains a doxylstearate labeled at carbon-12 of the fatty acid in PC, and this chemical side group quenches PM fluorescence, which is primarily static rather than collisional. Only when the doxyl moiety buried within the membrane bilayer is no more than 5 Å from PM-labeled cysteine can the fluorescence of PM be efficiently quenched [27]. Fig. 4C showed that the fluorescence intensity of the PM-labeled cysteine was sharply decreased by 12-DPC. This result indicated that tBid had the intrinsic potential to penetrate deeply into acidic phospholipid membranes rather than at a superficially shallow location.

4. Discussion

As a member of the BH3 domain only subset of Bcl-2 family, Bid is involved in various pathways of apoptosis that interplay the activation of caspases with mitochondrial dysfunction. A convergence of biochemical and cellular studies was committed to investigate the mechanism by which Bid acts on mitochondrial membranes [7,28]. To address this question, model membranes with definite compositions had been constructed to resemble the interaction of tBid with mitochondrial membranes, leading to cytochrome *c* release from mitochondria. A crucial point for understanding the interaction of tBid with model membranes is the determination of the possible mechanism of leakage. Through extensive studies on amphipathic α-helical peptides, researchers had proposed two general mechanisms underlying the release of entrapped probes: transmembrane pore formation with a barrel stave mechanism and membrane destruction with a carpet-like mechanism [20].

In this study, we used the fluorescent dextrans with various molecular diameters as the markers to test the leakage promoted by tBid in the model membrane systems. As shown in Fig. 1, following the action of tBid on the LUVs, FD-20, due to its smaller molecular diameter, released at a greater extent from LUVs than FD-70 or FD250S; tBid was more effective in promoting leakage of FITC-dextrans from LUVs composed of CL and DOPC compared with LUVs made of DOPA or DOPG and DOPC. This pertinency of the leakage

efficiency to the molecular diameters of fluorescent markers meant that discrete pores might be formed by the protein after its interaction with membranes. Otherwise, markers with different molecular weights would have released with the same efficiency if the carpet-like mechanism functioned [20,29]. In the leakage assay, with tBid concentration kept constant, increasing the lipid concentration resulted in a decrease in the extent of leakage, while decreasing the amount of tBid led to a reduction in leakage. This finding was also consistent with a monomer–multimer assembly pore-forming process. The pore-forming behavior of tBid in membranes might be accountable for the leakage of apoptotic factors from mitochondria, with molecular weights ranging from 14 kDa (cytochrome *c*) to 57 kDa (AIF) [5,30–32]. Moreover, displacement of DOPC by DOPE or DEPE decreased the leakage, and the leakage efficiency was further reduced with increasing amount of DOPE or DEPE in the membranes (Fig. 2). Notably, this phenomenon was also observed in the case of the pore-forming peptides such as GALA or magainin-2 [23,24]. It has been suggested that PE, with a smaller headgroup, would stabilize the surface state of proteins and hence reduce the protein insertion and subsequently pore formation [23]. This may account for our observation, further suggesting the pore-forming behavior of tBid. It is worthy to note that DOPE, characterized with non-lamellar phase tendency, showed no prompting effect on the action of tBid on the membrane in our observations. Thus, it can be questioned why CL, also known for its non-lamellar phase tendency, showed the specific role in tBid-induced leakage compared to DOPA and DOPG. This special property of CL in the interaction with tBid deserves further investigation to shed light on its biological roles.

The pore-forming mechanism involves three distinct steps. First, monomers bind to the membrane; second, these monomers insert into the hydrophobic core of the lipid bilayer; and finally, pores form in the membranes. To investigate if tBid actually inserted into the hydrophobic core of the membrane, the phospholipid monolayer assay and fluorescence quenching measurement were carried out (Figs. 3 and 4). The data showed that tBid did insert deeply into the hydrophobic acyl chain of acidic phospholipids, rather than merely binding at the surface of LUVs or being located at a superficially shallow position.

Recent studies showed that the insertion of tBid into the mitochondrial membrane might cause a conformational change, such as the exposure of hydrophobic domains that were previously buried, that enabled oligomerization of tBid [19]. Moreover, the three-dimensional structure and the in vitro electrophysiological data also suggested tBid as a pore-forming protein [11,17,18]. Through model membrane studies, we tentatively proposed that tBid could penetrate into the hydrophobic core of the membrane, resulting in the leakage of entrapped FITC-dextrans from LUVs more likely by a pore-forming mechanism. It can further be speculated that tBid oligomers might form pores in the mitochondrial membrane. More evidence in vivo is also required to clarify the rationality of this model. Related studies are still in progress.

Acknowledgements: This work was financially supported by grants from the National Natural Science Foundation of China and the Chinese Academy of Sciences. We thank Dr. Xiaodong Wang, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA for the generous gift of caspase 8 expression plasmids. We thank Dr. Bruno Antonsson, Serono Pharmaceutical Research Institute, Switzerland for the murine Bid expression plasmid. We are grateful for the assistance of Yunfeng Li in the phospholipid monolayer assay.

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