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Interactions of Bax and tBid with Lipid Monolayers

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Abstract. The release of cytochrome c from mitochondria to the cytosol is a crucial step of apoptosis that involves interactions of Bax and tBid proteins with the mitochondrial membrane. We investigated Bax and tBid interactions with (i) phosphatidylcholine (PC) monolayer as the main component of the outer leaflet of the outer membrane, (ii) with phosphatidylethanolamine (PE) and phosphatidylserine (PS) that are present in the inner leaflet and (iii) with a mixed PC/PE/Cardiolipin (CL) monolayer of the contact sites between the outer and inner membranes. These interactions were studied by measuring the increase of the lipidic monolayer surface pressure induced by the proteins. Our measurements suggest that tBid interacts strongly with the POPC/DOPE/CL, whereas Bax interaction with this monolayer is about 12 times weaker. Both tBid and Bax interact moderately half as strongly with negatively charged DOPS and non-lamellar DOPE monolayers. TBid also slightly interacts with DOPC. Our results suggest that tBid but not Bax interacts with the PC-containing outer membrane. Subsequent insertion of these proteins may occur at the PC/PE/CL sites of contact between the outer and inner membranes. It was also shown that Bax and tBid being mixed in solution inhibit their insertion into POPC/DOPE/CL monolayer. The known 3-D structures of Bax and Bid allowed us to propose a structural interpretation of these experimental results.

Key words: Bax — tBid — Lipid monolayers — Apoptosis — Three-dimensional structure

Introduction

Apoptosis is a genetically controlled cell death involved in the embryonic development, in homeostasis and also in human disease like cancer. A crucial step of this event is the release of cytochrome c from the intermembrane mitochondrial space to the cytosol. In order to elucidate the mechanism of permeabilization of the outer mitochondrial membrane responsible for the cytochrome c transfer, proteic or proteolipid pores induced by cytosolic Bax, tBid proteins and lipids were proposed. tBid is the carboxy-terminal portion obtained after cleavage by caspase 8 of the inactive cytosolic Bid protein. The specificity of tBid with anionic lipids was shown by Lutter et al. (2000). Interactions between Bax, tBid and lipids were studied in liposomes, proteoliposomes (Antonsson et al., 1997; Lutter et al., 2000; Saito et al., 2000; Roucou et al., 2002a; Yethon et al., 2003; Zamzami & Kroemer, 2003; Epand et al., 2004; Oh et al., 2005) and plane lipid bilayers (Basanez et al., 1999; Schendel et al., 1999). The role of lipid membranes in the cell death process has been recently summarized in a review (Cristea & Degli, 2004). Despite this progress the sequence of events and the details of the molecular mechanism of the process that involves Bax, tBid and lipids, and that results in the release of cytochrome c, are unknown. Here we study the penetration of these proteins into lipid monolayers. Such a model allows the analysis of the protein-lipid interactions in an interfacial organization similar to a biological membrane, under controlled parameters, mainly lateral pressure and lipidic and subphase composition (Brockman, 1999). Concerning the lipidic composition, we study the interactions between proteins and DOPC, the main component of the outer leaflet of the outer mitochondrial membrane; this leaflet is the first barrier that cytosolic proteins have to cross in order to penetrate into the mito-

Abbreviations CL, Cardiolipin; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; POPC, palmitoyloleoylphosphatidylcholine

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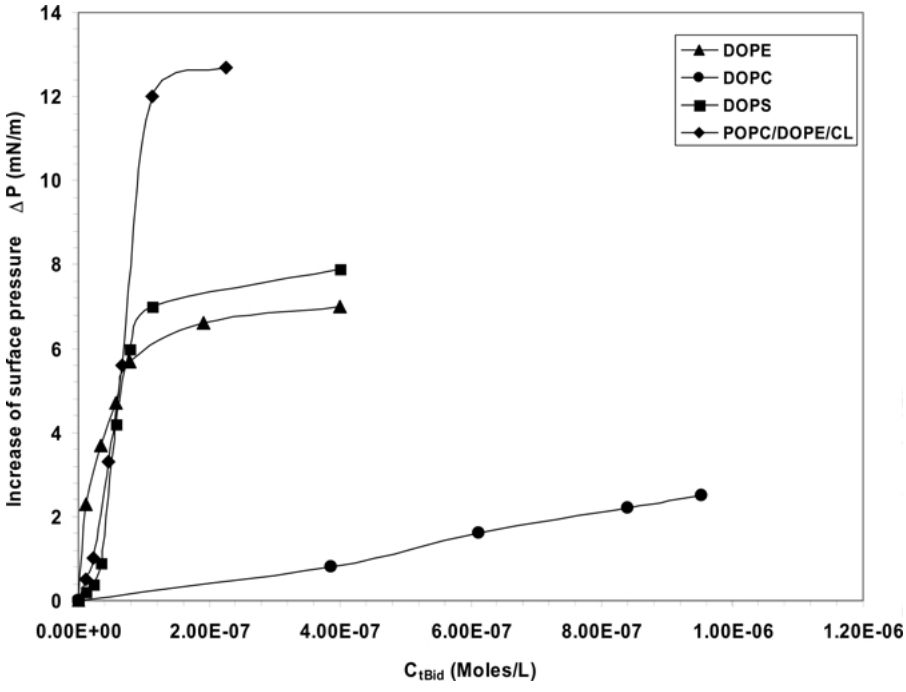

Fig. 1. Adsorption of tBid on phospholipid monolayers. The subphase is a HEPES (25 mM/L) buffer solution, with pH = 7.5, and $CaCl_2$, 8 mM/L, The lipidic composition of the monolayer is: ♦POPC/DOPE/CL with a molar fraction ratio of 1/1/1; ● DOPC; ▲ DOPE; and ■ DOPS; the initial surface pressure of the phospholipid monolayer is 30 mN/m.

chondrial membrane. We also investigate interactions of proteins with negatively charged DOPS and CL and nonlamellar DOPE lipids. These lipids are found in contact sites of the inner leaflet of the outer membrane with the inner membrane.

Materials and Methods

A lipid monolayer was made by spreading aliquots of a chloroform/methanol solution of lipid on an aqueous solution; the molecular density of spread lipid was calculated to induce a determined initial surface pressure of 30 mN/m, a lateral pressure expected for a cell membrane (Demel et al., 1975). After evaporation of the volatile solvent, lipidic amphipathic molecules orient themselves into a monomolecular film. Aliquots of aqueous solution of proteins were injected beneath the lipid monolayer, into the aqueous subphase maintained at a pH of 7.5 with HEPES buffer. Homogeneity of the subphase was provided by gentle magnetic stirring. The increase of lipid monolayer surface pressure consecutive to the insertion of proteins was measured with the Wilhelmy plate method (Adamson, 1990) using a Prolabo tensiometer (Paris, France), connected to an X/Y/t recorder (model BD 91, Kipp and Zonen, Delft, The Netherlands). The experiment was performed in a controlled atmosphere of argon to avoid oxydation of unsaturated lipids.

In this study, in order to see the effect of the phospholipid polar heads, we analyzed the interaction of proteins with pure neutral double-unsaturated dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and pure unsaturated negative dioleoylphosphatidylserine (DOPS). A mixture of palmityloleoylphosphatidylcholine (POPC), DOPE and cardiolipin (CL) with molar ratio (1/1/1) was also used.

Natural bovine heart CL was from Sigma (St Louis, MO), whereas synthetic POPC, DOPC, DOPE, DOPS were from Avanti Polar (Alabaster, AL). Human Bax, Bid and tBid proteins were produced in the laboratory of J.C. Martinou (Genéve, Switzerland) according to Roucou et al. (2002b).

Results

tBid Interaction with Lipid Monolayers

Apoptotic proteins act on the outer mitochondrial membrane (von Ahsen et al., 2000) and more precisely on contact sites between the inner and the outer mitochondrial membranes, which are mainly composed of neutral unsaturated phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL), with approximate molar ratio (1/1/1) (Ardail et al., 1990; Simbeni et al., 1991; Schlame et al., 2000; Lutter et al., 2001). We studied interaction of tBid with the following monolayers: (i) a mixture of POPC/DOPE/CL with molar ratio (1/1/1), which mimics well a lipidic composition in the contact sites; (ii) DOPC monolayer, which corresponds to the outer leaflet, (iii) DOPS monolayer, which is abundant in the inner leaflet and (iv) DOPE monolayer, which is also present in the inner leaflet (Fig. 1). tBid injection beneath these lipid monolayers induces an increase of their surface pressure in the following order: DOPC/DOPE/CL > DOPE ≅ DOPS > DOPC. An increase of surface pressure upon addition of a given molecule usually implies that this type of molecule interacts with the lipid monolayers. At the same time, this can be either an interaction with the polar heads of the lipid surface or penetration into the non-polar environment of the monolayer. Therefore, the use of the pressure increase data obviously does not distinguish well between surface binding and penetration. Previous experiments suggested that the addition of molecules that penetrate into the membrane causes a strong increase (over 5

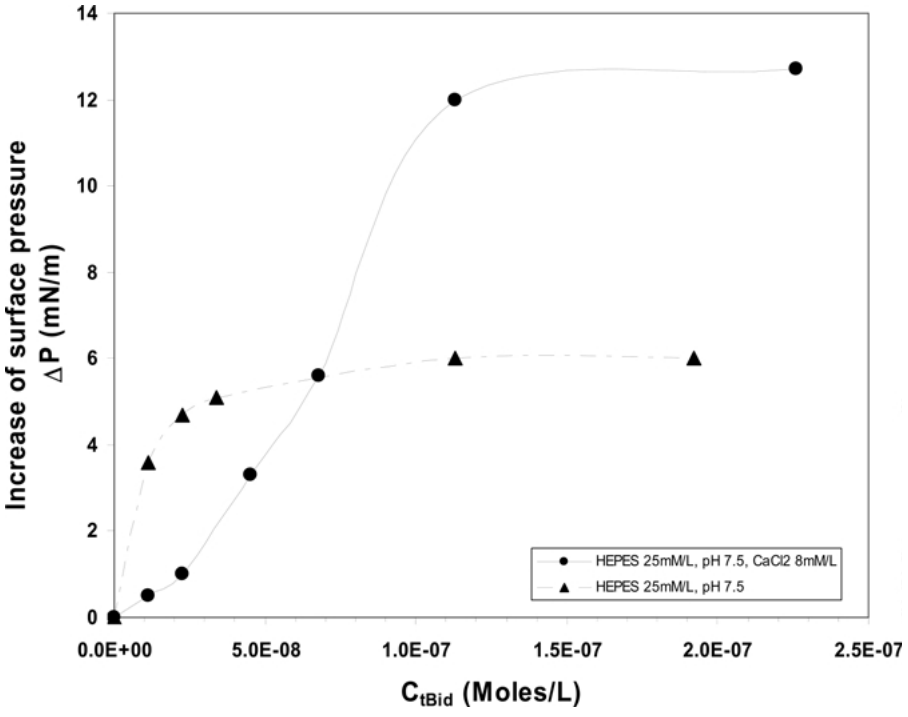

Fig. 2. Effect of the calcium ion on the tBid adsorption on a mixed monolayer POPC/DOPE/CL (1/1/1). The initial surface pressure of the phospholipid monolayer is 30 mN/m. The subphase is a HEPES (25 mM/L) buffer solution, with pH = 7.5, ● with $CaCl_2$ 8 mM/L and ▲ without $CaCl_2$.

mN/m at 30 mN/m initial lipid surface pressure) (Campagna et al., 1999; Vie et al., 2001), while hydrophilic proteins that bind the surface of the lipid membrane induce a weak (less than 5 mN/m) increase of surface pressure. The surface pressure may also depend on the cross-sectional area of the membrane-penetrating portion of the protein. Therefore, in our interpretation of the observed change of surface pressure upon injection of Bax and tBid we used these criteria, assuming that these molecules would have an unchanged cross-sectional area upon penetration into the lipid monolayer. Our study showed that the increase of surface pressure induced by the injection of tBid beneath the POPC/DOPE/CL monolayer reaches 12 mN/m at a protein concentration of 2×10^{-7} M. Such an increase allows us to suppose that tBid inserts into the lipid monolayer and interacts with the hydrophobic chains of lipids. In contrast, an experiment with a full-length Bid does not show any interaction with this monolayer (*data not shown*). The experiment also shows that the presence of tBid significantly increases the surface pressure of negatively charged DOPS. Almost the same increase was recorded for the monolayer of DOPE (Fig. 1), which is a neutral lipid with a small polar head that can self-assemble into monolayers with negative curvature and bilayers with local bulging effect (Chernomordik et al., 1985). The smallest but still noticeable increase of surface pressure was observed in the case of DOPC monolayers (2 mN/m at the maximal tested concentration of tBid 9×10^{-7} M). Based on the fact that monolayers with a surface pressure of 30 mN/m mimic well bilayers of the same lipid composition (Demel et al., 1975), we can conclude that tBid can insert spontaneously into lipid bilayers with a specificity for DOPE and negative lipids (CL and DOPS). This result agrees with (Oh et al., 2005) who observed a better affinity of tBid for negative CL and PG lipids than for neutral ones.

Effect of Calcium on the Interaction of Tbid With Lipid Monolayers

Taking into account that the presence of bivalent cations enhances the permeability of membranes induced by proapoptotic proteins (Epand et al., 2004), we studied the effect of calcium ion on the insertion of tBid into POPC/DOPE/CL monolayers. $CaCl_2$ was added into the subphase aqueous solution at a concentration of 8 mM. This concentration was previously used to evidence the formation of pores induced by Bax in liposomes (Epand et al., 2002b). Then, similarly to the experiments without calcium, we recorded surface pressure as a function of protein concentration.

Our measurements show that at tBid concentrations below 7×10^{-8} moles /L, the increase of surface pressure of the lipid monolayer is lower in the presence of calcium than without it (Fig. 2). Inversely, for higher tBid concentrations, the surface pressure is higher with Ca^{2+} ion than without it. A possible explanation of this result is that at low tBid concentrations Ca^{2+} ions inhibit adsorption of tBid due to a screen effect, whereas the high tBid concentrations favor oligomerization of tBid in solution and tBid oligomers can more easily insert

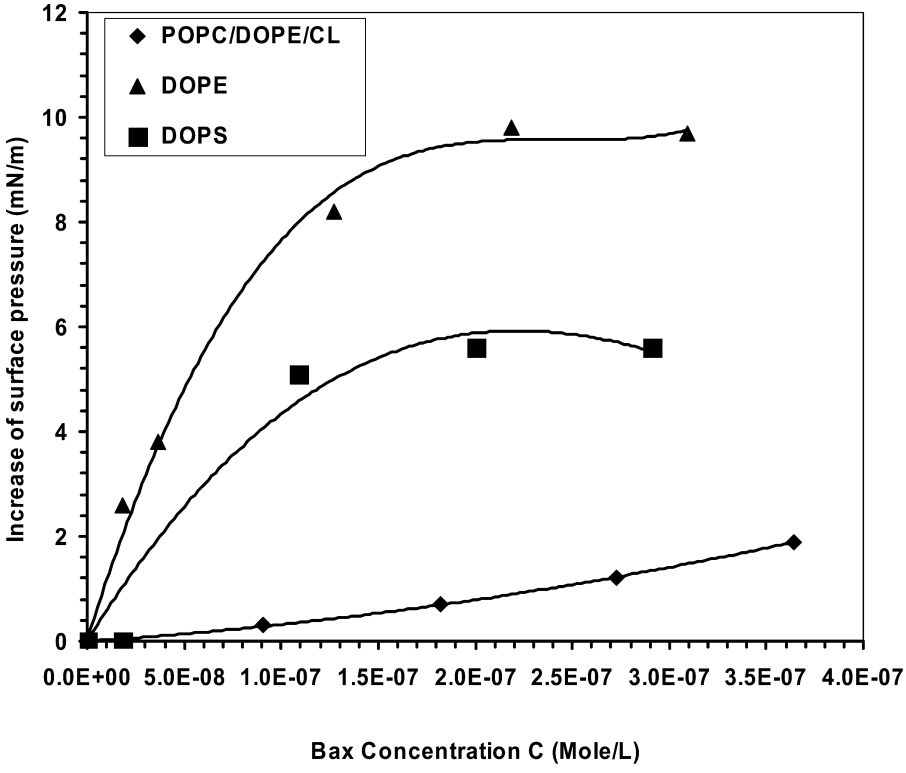

Fig. 3. Adsorption of Bax on phospholipid monolayers. The subphase is a HEPES (25 mM/L) buffer solution, with pH = 7.5, and $CaCl_2$, 8 mM/L, The lipidic composition of the monolayer is: ♦ POPC/DOPE/CL with a molar fraction ratio of 1/1/1; ▲ DOPE; and ■ DOPS; the initial surface pressure of the phospholipid monolayer is 30 mN/m.

into the lipid monolayer than the monomers. The difference in surface pressure at saturating tBid concentration can also be explained by the Ca^{2+}-induced conformational change in tBid, which may lead to a structure covering more lipids per protein in the presence of calcium.

Bax Interaction with Lipid Monolayers

Figure 3 shows the surface pressure of a mixed monolayer POPC/DOPE/CL (at a molar ratio 1/1/1) starting from 30 mN/m, as a function of Bax monomer concentration into the subphase. A small (12 times less than tBid) increase of surface pressure is observed. Assuming that the increase in surface pressure per inserted tBid or Bax molecule is the same, we can conclude that Bax interaction with the monolayer is about 12 times weaker at concentrations up to 4×10^{-7}M than that of tBid. The low value of the increase (about 2 mN/m up to 4×10^{-7}M) suggests a weak adsorption of this protein on the lipid monolayer that can be due to weak interactions between the phospholipid polar heads and Bax.

Similarly to tBid, the influence of lipid polar heads on Bax adsorption was investigated by using pure negatively charged DOPS, neutral DOPE and DOPC. A high increase of surface pressure is observed with the negative unsaturated lipid DOPS, as well as the neutral DOPE. In contrast, we did not observe any pressure increase, that is, any penetration of Bax into DOPC (*data not shown*). These results show that Bax does not interact with the main lipid of the outer leaflet but interacts with lipids found in contact sites and in the inner leaflet, namely, negatively charged lipid DOPS and non-lamellar lipid DOPE.

Interaction of Bax - tBid Mixture with Lipid Monolayers

Previously it has been reported that Bax monomer has no apoptotic activity and must be in an oligomerized state to induce cytochrome c release. It has also been indicated that tBid is necessary but not sufficient (Roucou et al., 2002a) to form homo- or heterodimers of Bax (Desagher et al., 1999; Antonsson et al., 2000; Eskes et al., 2000). Therefore, we coincubated tBid and Bax at a total protein concentration of 2×10^{-7} mole/L, in a molar ratio of 1/5; this ratio was used previously in order to induce the release of cytochrome c in purified brain mitochondria (S. Desagher, personal communication). This mixture of proteins yields an increase of surface pressure of the monolayer POPC/DOPE/CL that is smaller than one recorded with the addition of the same amount of proteins separately (Fig. 4). This result indicates that, in solution, Bax is not activated by tBid. This result agrees with several studies that suggest the ability of Bax to oligomerize independently of the presence of tBid (Korsmeyer et al., 2000; Epand et al., 2002b; Roucou et al., 2002a; Yethon et al., 2003). In order to verify whether a heterodi-

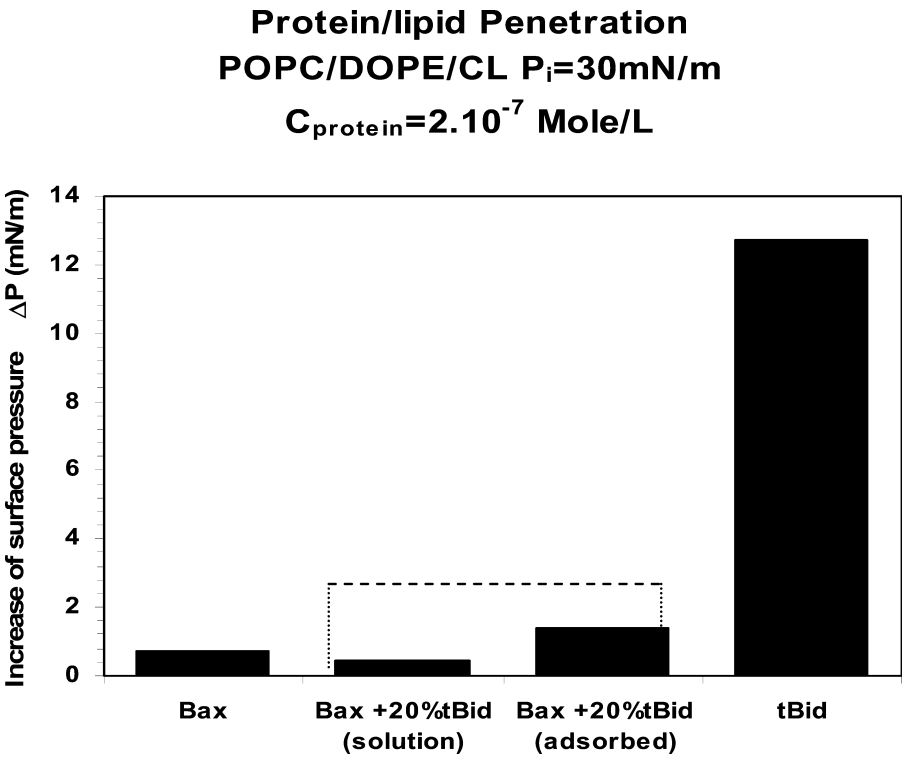

Fig. 4. Comparison of insertions of Bax and tBid mixtures of different compositions on a POPC/DOPE/CL (1/1/1) monolayer with an initial pressure of 30 mN/m; the subphase composition is HEPES (25 mM/L) buffer solution, with pH = 7.5, $CaCl_2$ 8 mM and the concentration of the injected protein is 2×10^{-7} M. The dotted line indicates the surface pressure that would be obtained with additivity of the two components.

merization takes place on the water/lipid interface, we first injected Bax and when the surface pressure reached equilibrium, we added t-Bid at the ratio 1/5. Then, Bax and tBid were added and this operation was repeated several times until the protein concentration reached 2×10^{-7} mole/L. The increase of surface pressure of the lipid monolayer induced by pure and mixed proteins at the concentration of 2×10^{-7} mole/L is shown in Fig. 4. The dotted line indicates the value of ΔP, which would be obtained with tBid and Bax at a total protein concentration of 2×10^{-7} mole/L and a molar ratio of 1/5 like in the mixture. It was observed that in these cases the increase of the monolayer surface pressure was always smaller than with the addition of each protein separately. Thus, coincubation of Bax and tBid inhibits their adsorption into the lipid monolayers.

Discussion

Insertion of tBid into Lipid Monolayers

Our experiments suggest that tBid inserts spontaneously into monolayers composed of lipids having various polar heads. The weak increase of DOPC surface pressure (the main component of the outer leaflet of the outer mitochondrial membrane) indicates that tBid may be located near the polar heads, carpeting the monolayer like antimicrobial peptides (Zasloff, 2002) and can destabilize the outer leaflet, inducing strains within the bilayer. Monolayers consisting mostly of negatively charged lipids (DOPS and POPS/DOPE/CL) are especially favorable for tBid insertion. The high increase of surface pressure suggests that tBid inserts into these lipid monolayers. These results are in agreement with recent data obtained by a site-directed spin-labelling study (Oh et al., 2005) suggesting that tBid is located near the hydrophobic chains between a polar and a nonpolar environment. This result is also in agreement with those of Yan et al. (2003) who observed a higher critical pressure induced by tBid in the anionic phospholipid monolayers than in the neutral ones. It also agrees with an observation that tBid forms channels in plane lipid bilayers (Schendel et al., 1999) and destabilizes lipid membranes (Schendel et al., 1999; Kudla et al., 2000), both having negative lipids. The preference of tBid interaction with negative lipids was also evidenced by Lutter et al. (2000). Thus, we can conclude that tBid can insert into the mitochondrial outer membrane near the contact sites between the inner and the outer mitochondrial membrane where negatively charged lipids are located (Lutter et al., 2001).

It has previously been shown that in physiological conditions negatively charged CL decreases cohesive energy between the two leaflets of a membrane (Nichols-Smith et al., 2004) and tBid induces a negative curvature of lipid bilayers (Epand et al., 2002a). These effects together with the ability of tBid to insert into lipid monolayers can favor the inner leaflet expansion of the bilayer and the lipidic pore formation. The observed increase of surface pressure

induced by tBid in the neutral DOPE monolayer may be associated with a modification of the negative curvature of the monolayer in presence of this protein.

Interaction of Bax with Lipid Monolayers

In contrast to tBid, Bax does not interact with neutral DOPC, the main component of the outer leaflet of the outer mitochondrial membrane. Bax has a weak interaction with the POPC/DOPE/CL monolayer (Fig. 3), while negatively charged DOPS monolayer interacts with Bax equally well as with tBid. Interestingly, these results differ from those described by Yethon (2003) who interpreted the change in and stabilization of Bax conformation in the presence of liposomes as an interaction between Bax and liposomes of different lipidic composition.

The 3-D structure of Bax (Suzuki et al., 2000) has a small cluster of positively charged side chains (formed by residues 34, 37, 57, 64, 65, 78, 119, 123 and 128) that can explain the adsorption of Bax by negatively charged lipids. Similarly to tBid, Bax also interacts well with non-lamellar DOPE monolayers. The penetration into DOPE seems to be contradictory to the previously published results (Basanez et al., 2002) that non-lamellar lipids affect liposome permeabilization induced by Bax, but that lipids with intrinsic negative curvature, like DOPE, diminish the permeabilization. However, this study was realized on bilayers formed by either non-lamellar lipid octyllyso-phospholipid (O-LP) exhibiting positive curvature or DOPE with negative curvature, whereas in our work we compare DOPC monolayer forming non-lamellar structure and DOPE monolayer forming non-lamellar structure with negative curvature. Our results are in accordance with the observation that Bax monomers being in solution destabilize membranes, form pores in the vesicules (Saito et al., 2000) and destabilize planar bilayers (Basanez et al., 1999, 2001) containing negatively charged lipids.

When trying to correlate our results with the situation in vivo, it is important to underline that Bax, in contrast to tBid, does not interact with monolayers of DOPC, which is the main component of the outer leaflet of the outer mitochondrial membrane. Bax only inserts into DOPS and DOPE monolayers located on the inner leaflet of this membrane, which is not accessible for the cytosolic proteins. Our results suggest that cytosolic Bax cannot spontaneously insert into the outer lipidic leaflet of the mitochondrial membrane and needs an additional mediator. Along this line, recently Terrones et al. (2004) reported that tBid assists Bax via protein-lipid interactions. Other data (Enoksson et al., 2004) suggest the role of caspase 2 acting in conjunction with Bax/Bak to amplify cytochrome c release by causing its displacement from cardiolipin.

Insertion of Mixed Proteins into Lipid Monolayers

When Bax and tBid are coincubated in solution before injection beneath the lipid monolayer or when they are injected one after the other, the increase of the monolayer surface pressure is always less than when proteins are injected separately at identical concentrations. This suggests that tBid interacts with Bax in the aqueous subphase (Desagher, 1999) beneath the monolayers and inhibits Bax and tBid insertion into the lipid monolayer.

In the case of a lipid bilayer, however, we cannot exclude that the adsorption of tBid near the polar heads of neutral lipid DOPC (the main component of the outer leaflet of the outer membrane) may induce transbilayer lipid diffusion as it was previously observed in another study (Epand et al., 2003). This diffusion may dislocate negatively charged lipids on the outer leaflet and allow their interaction with Bax protein.

Structural Interpretation

The analysis of the known 3-D structures of Bax and Bid (McDonnell et al., 1999) allows us to gain insight into the possible molecular mechanisms of interactions between lipids and tBid and Bax proteins. The analysis of the 3-D structure of Bid indicates that truncation of its N-terminal region, which transforms it to tBid, should eliminate a significant part of the structure where the BH3 helix is nested and this helix should become more mobile (BH1, BH2 and BH3 are the homology regions of the Bcl-2-like proteins). The detachment of the BH3 helix from the core structure creates two new hydrophobic surfaces with a high potential for interaction with other molecules. One of them is the BH3 helix itself, another one is a surface partially formed by the BH1 and BH2 regions (Fig. 5). Therefore, it is reasonable to suggest that tBid will insert into the lipid monolayers either by the amphiphilic BH3 helix or by the BH1-BH2 site (Fig. 5). The result of a recent site-directed spin-labelling study favors the latter case (Oh et al., 2005). The BH1-BH2 site is composed not only of apolar side chains (residues 155, 156, 177, 188, 190, 191 and 196) but also has a cluster of positively charged side chains (residues 159, 160, 170, 185, 189, 193). This can explain an easier insertion of tBid into the negatively charged lipid monolayers.

A mixture of tBid and Bax in solution inhibits their insertion and this can be explained by interaction of these proteins. Indeed, it was shown that Bax and tBid interact with each other (Desagher et al., 1999). The recently determined structure of the complex between Bim (an analog of tBid) and Bcl-x_L (structurally similar to Bax) (Liu et al., 2003) suggests

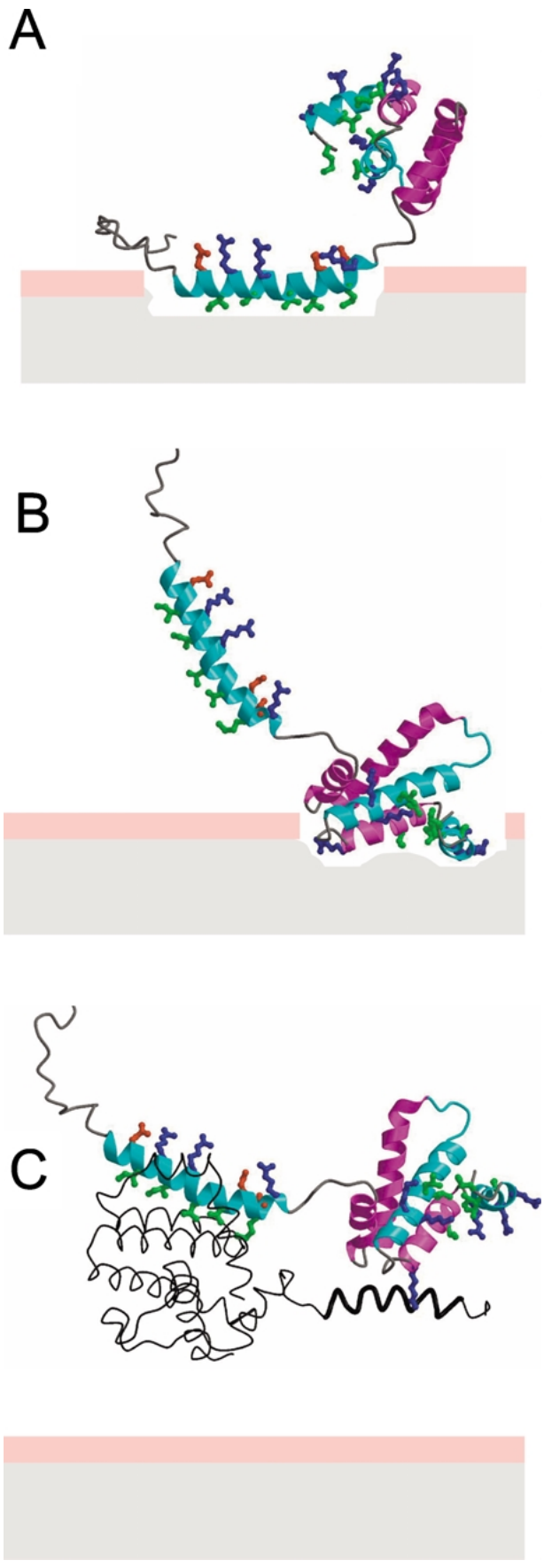

Fig. 5. Schematic diagrams showing possible interactions of tBid with lipid monolayers and with Bax. In the ribbon representation of tBid, BH1, 2 and 3 helices are in green. The trace of the Bax molecule is shown by a thin line, while the TM helix of Bax is outlined by the thick line. Side chains of tBid surfaces with a potential for intermolecular contacts are shown as balls-and-sticks. Hydrophobic side chains are in green, positively charged side chains are in blue and negatively charged ones in red. Layers of polar heads of lipids are in pink. (*A*) BH3 of tBid interacts with the lipid monolayer. (*B*) BH1, BH2 surface of tBid inserts itself into the lipid monolayer. (*C*) Interactions of Bax and tBid in solution.

how tBid and Bax can interact with each other in solution, forming a tBid-Bax complex that is not permissible for interaction with the lipids. In the known complex, the BH3 helix of Bim interacts with the pocket of Bcl-x_L which is normally a binding site for the transmembrane (TM) helix of Bcl-x_L. This allows us to assume that similarly, the BH3 helix of tBid may compete with TM of Bax for this binding site and the displaced TM of Bax can interact with the BH1-BH2 site of tBid (Fig. 5). Such a swapping can prevent the tBid-Bax complex from insertion into the lipid monolayer.

In accordance with our results, the full-length Bid molecule does not interact with lipids. This agrees with the above interpretation, because in the Bid structure the hydrophobic and positively charged helices BH3, BH1 and BH2 are in contact with each other and not free for intermolecular interactions. We also demonstrated that Bax in a cytosol-like solution have weak interaction with the lipid monolayers. Similarly to the full-length Bid, the amphipathic TM helix of Bax molecules forms intramolecular contacts (Roucou & Martinou, 2001) and is not free to interact with the membrane. Preference of Bax to the negatively charged lipids can be explained by presence of a small cluster of positively charged residues on the surface of Bax.

In conclusion, our results obtained by measuring the increase of the lipidic monolayer surface pressure induced by the proteins Bax and tBid support the concept that during the process of apoptosis, tBid interacts with lipids, whatever their nature. It preferentially inserts into negative lipids of the mitochondria contact sites between the inner and outer membranes, whereas Bax does not spontaneously insert into PC, the main component of the outer lipidic leaflet of the mitochondrial membrane. At the same time, binding of tBid to the membrane may initiate insertion of Bax and by doing this constitute the first step of formation of pores leading to apoptosis (Chanturiya et al., 2004).

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