Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein

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In response to various apoptotic stimuli, Bax, a pro-apoptotic member of the Bcl-2 family, is oligomerized and permeabilizes the mitochondrial outer membrane to apoptogenic factors, including cytochrome *c*. Bax oligomerization can also be induced by incubating isolated mitochondria containing endogenous Bax with recombinant tBid (caspase-8-cleaved Bid) *in vitro*. The mechanism by which Bax oligomerizes under these conditions is still unknown. To address this question, recombinant human full-length Bax was purified as a monomeric protein. Bax failed to oligomerize spontaneously in isolated mitochondria or in liposomes composed of either cardiolipin or lipids extracted from mitochondria. However, in the presence of tBid, the protein formed large complexes in mitochondrial membranes and induced the release of cytochrome *c*. tBid also induced Bax oligomerization in isolated mitochondrial outer membranes, but not in other membranes, such as plasma membranes or microsomes. Moreover, tBid-induced Bax oligomerization was inhibited when mitochondria were pretreated with protease K. The presence of the voltage-dependent anion channel was not required either for Bax oligomerization or for Bax-induced cytochrome *c* release. Finally, Bax oligomerization was reconstituted in proteoliposomes made from mitochondrial membrane proteins. These findings imply that tBid is necessary but not sufficient for Bax oligomerization; a mitochondrial protein is also required.

Key words: apoptosis, Bcl-2, cytochrome *c*, mitochondrial outer membrane.

INTRODUCTION

The mitochondrion plays a central role in apoptosis triggered by various stimuli [1,2]. Indeed, mitochondria sequester several apoptogenic proteins that are released into the cytosol during apoptosis. These proteins can be divided into two groups. The first group, comprising cytochrome *c*, Smac/Diablo and the serine protease Omi/HtrA2, regulates the activation of caspases. Once in the cytosol, cytochrome *c* induces the oligomerization of the cytosolic protein Apaf-1 (apoptotic protease-activating factor 1) [3] which, in the presence of ATP/dATP, recruits and activates procaspase-9 [4–6]. On the other hand, Smac/Diablo and Omi/HtrA2 bind to and inactivate inhibitor of apoptosis proteins (IAPs), which normally inhibit caspase-9 and -3 activity [7–11]. The second group of mitochondrial apoptogenic factors is composed of two proteins that trigger nuclear DNA cleavage: apoposis-inducing factor causes chromatin condensation and large-scale DNA fragmentation [12,13], whereas endonuclease G induces nucleosomal DNA fragmentation [14,15].

The Bcl-2 family of proteins, composed of anti-apoptotic and pro-apoptotic members, controls the release of mitochondrial apoptogenic proteins [16–18]. Members of the Bcl-2 family possess at least one of four conserved domains known as Bcl-2 homology (BH) domains (BH1–BH4) that regulate homo- and hetero-complex formation among the Bcl-2 family of proteins. The pro-apoptotic proteins are subdivided into two groups: multidomain proteins such as Bax and Bak, and BH3-domain-only proteins such as Bid. In normal cells, Bax and Bak are expressed, but they are not active. Bax is cytosolic [19], whereas Bak appears to be inserted in mitochondrial membranes in an inactive conformation [20,21]. Upon induction of apoptosis, both proteins undergo a conformational change and oligomerize in the mitochondrial outer membrane (MOM). In hepatocytes, these events are triggered by the BH3-domain-only protein Bid after its cleavage by caspase-8 into a 15.5 kDa C-terminal fragment (tBid), and are followed by the release of mitochondrial apoptogenic proteins. In favour of this mechanism, it was shown that cells lacking both Bax and Bak, but not cells lacking only one of these components, were completely resistant to Bid-induced cytochrome *c* release and apoptosis [22]. In contrast, anti-apoptotic members of the family, such as Bcl-2 and Bcl-X_L, prevent the release of mitochondrial apoptogenic factors [23,24]. This could occur in part by sequestration of BH3-domain-only proteins [25].

Several data indicate that only Bax or Bak oligomers are capable of permeabilizing MOMs. Following the discovery that the enforced dimerization of Bax resulted in mitochondrial dysfunction [26], it was demonstrated that Bax oligomers (purified in the presence of detergents that trigger Bax oligomerization [27,28]), but not monomers (purified in the absence of detergents), induced the release of cytochrome *c* from mitochondria [29]. It is now well established that Bax and Bak form large oligomeric complexes in apoptotic cells. Indeed, various apoptotic stimuli, such as staurosporine, tumour necrosis factor, UV treatment and ATP depletion, all induced oligomerization of Bax and/or Bak in mitochondria [30–33]. Fluorescence resonance energy transfer imaging of cultured cells revealed that Bax oligomerization is a prerequisite for cytochrome *c* release [34]. This oligomerization

Abbreviations used: Bax_{FL}, recombinant full-length human Bax; BH domain, Bcl-2 homology domain; $mBax_{FL}$, monomeric Bax_{FL}; MOM, mitochondrial outer membrane; $oBax_{FL}$, oligomeric Bax_{FL}; tBid, caspase-8-cleaved Bid; VDAC, voltage-dependent anion channel.

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can be reconstituted in isolated mitochondria containing endogenous Bax or Bak protein. Indeed, mitochondria isolated from HeLa cells contain some Bax protein loosely attached to the organelle. When added to these mitochondria, tBid induced Bax oligomerization in the outer membrane [35]. Similarly, tBid also induced Bak oligomerization in mitochondria isolated from hepatocytes [21]. Recently it was shown that tBid itself can also oligomerize in mitochondria and induce the release of cytochrome *c* [36]. The formation of oligomers correlates with the release of cytochrome *c* [35,37]. Therefore, elucidation of the mechanism of Bax and Bak oligomerization may help to determine the mechanism by which apoptogenic factors are released from mitochondria.

In the present study, we have addressed the mechanism of Bax oligomerization in mitochondria. We report that tBid is necessary, but not sufficient, for Bax oligomerization. A mitochondrial factor is also required.

MATERIALS AND METHODS

Yeast strains

Yeast strain M22-2 (Δ*por1*) has been described previously [38]. M3 is the *POR1* parent of M22-2.

Protein purification

Full-length human Bax (Bax_{FL}) with a tag of six histidine residues at the N-terminus was expressed in the pBAD plasmid in *Escherichia coli* [39]. Cells were lysed in the absence of any detergent, and monomeric Bax_{FL} ($mBax_{FL}$) was recovered in the soluble bacterial fraction, and purified by chromatography on nickel-nitriloacetic acid–agarose followed by Q-Sepharose. The protein was stored in 25 mM Tris/HCl, 100 mM NaCl, 0.2 mM dithiothreitol and 30 % (v/v) glycerol, pH 7.5, at −80 °C. Oligomeric Bax_{FL} ($oBax_{FL}$) was purified as previously described [39]. Mouse tBid (Bid cut with caspase-8) was obtained from full-length Bid as described previously [40].

Bax oligomerization in isolated mitochondria, microsomes, plasma membranes and MOM vesicles

Mitochondria were isolated from mouse or rat liver, or from yeast cells, as described previously [41,42]. Plasma membranes were isolated from rat erythrocytes. Briefly, 3 ml of whole blood was collected and thoroughly diluted to 10 ml in cold isotonic buffer (0.9 % NaCl, 5 mM sodium phosphate, pH 8). Erythrocytes were pelleted at 600 ***g*** for 10 min at 4 °C, and the cells were washed twice in the same buffer. The washed erythrocytes were lysed in 10 vol. of ice-cold 5 mM Na_2HPO_4, pH 8.0. Erythrocyte membranes were washed free of haemoglobin with this buffer by pelleting at 12000 ***g*** for 10 min at 4 °C. The wash cycle was repeated three times. Microsomes and MOM vesicles were isolated from rat liver [43]. Mitochondria, mitochondrial membranes or microsomal membranes (2 mg of protein) were added to the oligomerization buffer (125 mM KCl, 0.5 mM EGTA, 5 mM succinate, 10 mM Hepes/NaOH, pH 7.4, 4 mM $MgCl_2$, 5 mM Na_2HPO_4) in Eppendorf tubes coated with 0.4 % (w/v) BSA. When using membranes, 2 mM ATP was included in the oligomerization buffer. Recombinant $mBax_{FL}$ (50 nM) and tBid (5 nM) were added to mitochondria or membranes. After an incubation of 30 min at 30 °C, 0.11 ml of a solution of 20 % (w/v) CHAPS (in oligomerization buffer) was added. Solubilization was carried at 4 °C for 30 min. After centrifugation (10 min at 100000 ***g***), supernatants were analysed by gel filtration.

Bax oligomerization in proteoliposomes

Mitochondrial phospholipids were isolated from rat liver mitochondria by the method of Folch et al. [44]. Portions of 5 mg of mitochondrial phospholipids and 10 mg of phosphatidylcholine were dried under a stream of nitrogen and solubilized in 1 ml of oligomerization buffer. Lipids were sonicated for 5 × 30 s at 60 W, being left on ice for 1 min between rounds of sonication.

Rat liver mitochondria were diluted at a protein concentration of 5 mg/ml in a buffer containing 5 mM K_2HPO_4 and 5 mM EDTA, pH 7.2. They were incubated at 4 °C for 20 min, and sonicated five times with a metal probe at 60 W. After centrifugation (1 h at 100000 ***g***, 4 °C), the supernatant was discarded, and the membrane pellet was solubilized at a final protein concentration of 10 mg/ml in oligomerization buffer containing 4 % (w/v) Triton X-100. After an incubation of 30 min at 4 °C, the sample was centrifuged for 10 min at 100000 ***g*** and the supernatant was kept on ice.

Aliquots of 0.33 ml of solubilized membranes, 0.3 ml of liposomes and 0.37 ml of oligomerization buffer were added to 0.1 g of BioBeads SM2 (Calbiochem) that had been equilibrated previously with 1 ml of oligomerization buffer. The mixture was incubated for 1 h at room temperature and then centrifuged in order to pellet the BioBeads SM2. The supernatant was added to 0.1 g of fresh BioBeads SM2 equilibrated with 1 ml of oligomerization buffer, and incubated for 1 h at room temperature. BioBeads SM2 were removed by centrifugation. Proteoliposomes were transferred to a new tube and kept on ice. Proteoliposomes (0.3 ml) were added to an Eppendorf tube coated with 0.4 % (w/v) BSA. Bax oligomerization was tested as described above with mitochondria and membranes, in the presence of 2 mM ATP.

Gel filtration analysis

Gel filtrations were performed on a Superdex 200 (16/60) column from Amersham Pharmacia Biotech equilibrated in 25 mM Hepes/NaOH, pH 7.5, 300 mM NaCl, 0.2 mM dithiothreitol and 2 % (w/v) CHAPS, at a flow rate of 1 ml/min at 4 °C. The sample was loaded on to the column, and fractions of 2 ml were collected and analysed by Western blotting using a monoclonal antibody directed against Bax (2d2; Upstate Biotechnology).

Bax insertion and cytochrome *c* release

Mitochondria (0.2 mg) were added to 0.2 ml of release buffer (210 mM mannitol, 70 mM sucrose, 10 mM Hepes/NaOH, pH 7.4, 0.5 mM EGTA, 5 mM succinate, 4 mM $MgCl_2$ and 5 mM Na_2HPO_4). $oBax_{FL}$ (100 nM) or $mBax_{FL}$ (100 nM) was added in the absence or the presence of tBid (1 nM), and the mitochondria were incubated for 30 min at 30 °C. When testing for Bax insertion, mitochondria were centrifuged for 10 min at 10000 ***g***. Supernatants were discarded and pellets resuspended in 0.2 ml of 0.1 M sodium carbonate, pH 11.5. After an incubation of 20 min at 4 °C, samples were centrifuged for 10 min at 100000 ***g***, and the pellets were analysed by Western blotting using the anti-Bax monoclonal antibody 2d2. When testing for the release of cytochrome *c*, mitochondria were centrifuged for 10 min at 10000 ***g***. The presence of cytochrome *c* was analysed in both the supernatants and the pellets by Western blotting using a polyclonal antibody.

Cytochrome *c* efflux from liposomes was measured as described previously [45].

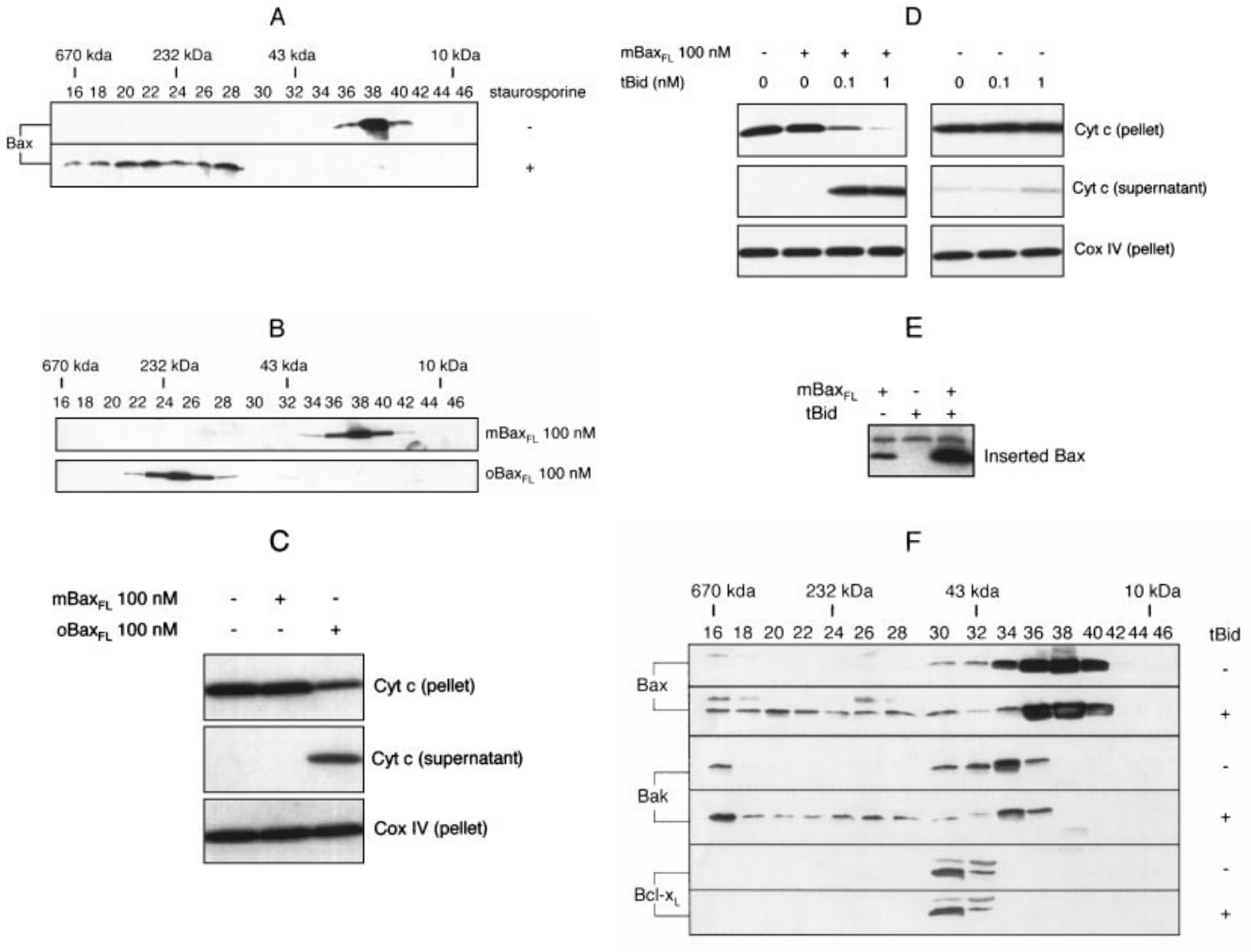

Figure 1 Reconstitution of Bax oligomerization in isolated mitochondria

(**A**) Mitochondria were isolated from untreated HeLa cells (−) and cells treated with 0.5 μM staurosporine (+). Proteins were solubilized with 2% (w/v) CHAPS, and fractionated on a Superdex 200 column (16/60). Fractions of 2 ml were collected, and every second fraction was analysed by SDS/PAGE and Western blotting with antibodies against Bax. (**B**) Recombinant Bax was purified from *E. coli* as monomers or as oligomers, as described in the Materials and methods section. Portions of 20 μg of the mBax$_{FL}$ and oBax$_{FL}$ proteins were analysed by gel filtration, and every second fraction was analysed by Western blotting with an antibody directed against Bax. (**C**, **D**) Mouse liver mitochondria were incubated with mBax$_{FL}$ or oBax$_{FL}$ in the absence or in the presence of 1 nM tBid for 30 min at 30 °C. After centrifugation, the presence of cytochrome *c* (Cyt c) was analysed in the supernatant and pellet fractions by Western blotting with a polyclonal antibody. Cytochrome *c* oxidase subunit 4 (Cox IV) was used as a gel-loading control for the pellet. (**E**) Mitochondria isolated from mouse liver were incubated with 100 nM mBax$_{FL}$ in the absence or in the presence of 1 nM tBid for 30 min at 30 °C. They were recovered by centrifugation and treated with 0.1 M Na_2CO_3. After centrifugation, inserted Bax was analysed by Western blotting of the membrane fraction. (**F**) Mitochondria were isolated from mouse liver and incubated with 50 nM mBax$_{FL}$ in the presence or in the absence of 5 nM tBid for 30 min at 30 °C. Proteins were solubilized and fractionated as described for (**A**). Fractions were analysed by Western blotting with antibodies directed against Bax, Bak and Bcl-X$_L$. Similar results were obtained in three independent experiments.

RESULTS

Reconstitution of Bax oligomerization in isolated mitochondria

Bax forms large oligomeric complexes in mitochondria from apoptotic cells [30]. This finding was confirmed in the present study. Mitochondria were isolated from untreated HeLa cells and from HeLa cells treated with staurosporine, and mitochondrial proteins were fractionated by gel filtration. As shown in Figure 1(A), Bax extracted from mitochondria isolated from untreated cells was eluted as a monomer, whereas it formed mainly large complexes in mitochondria isolated from apoptotic cells.

In order to study the mechanism of Bax oligomerization, the human protein was produced as a recombinant full-length monomeric (mBax$_{FL}$) or oligomeric (oBax$_{FL}$) protein. mBax$_{FL}$ was prepared in the absence of any detergent, while oBax$_{FL}$ was purified in the presence of Triton X-100, which artificially induces Bax oligomerization [27–29]. As shown in Figure 1(B), mBax$_{FL}$ and oBax$_{FL}$ can be easily separated by gel filtration. The ability of both mBax$_{FL}$ and oBax$_{FL}$ to permeabilize the MOM to cytochrome *c* was tested using mitochondria isolated from mouse liver. These mitochondria do not contain endogenous Bax (results not shown). mBax$_{FL}$ did not induce the release of cytochrome *c* (Figure 1C). However, addition of oBax$_{FL}$ permeabilized these mitochondria to cytochrome *c* (Figure 1C), confirming previous results obtained with truncated Bax lacking the C-terminal domain [29]. Therefore oBax$_{FL}$, but not mBax$_{FL}$, is able to induce the release of cytochrome *c* from isolated mitochondria.

Next we tested whether mBax$_{FL}$ could induce the release of cytochrome *c* in the presence of tBid. Recombinant Bid was

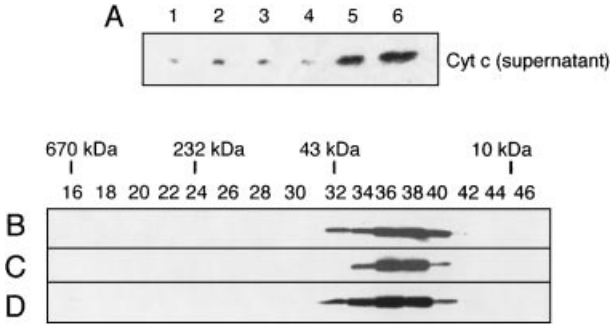

Figure 2 tBid does not induce the release of cytochrome *c* from liposomes, and does not induce Bax oligomerization in liposomes or in mitochondria treated with protease K

(**A**) Liposomes containing cytochrome *c* (Cyt c) were incubated in the absence of any recombinant protein (lane 1), or with 100 nM $mBax_{FL}$ (lane 2), 5 nM tBid (lane 3), 100 nM $mBax_{FL}$ plus 5 nM tBid (lane 4), 100 nM $oBax_{FL}$ (lane 5) or Triton X-100 to release all of the cytochrome *c* content (lane 6). After incubation for 10 min, vesicles were centrifuged and cytochrome *c* was detected in the supernatant by Western blotting. (**B**, **C**) Liposomes were incubated with 50 nM $mBax_{FL}$ and 5 nM tBid for 30 min at 30 °C. Bax oligomerization was analysed by gel filtration as described in the legend of Figure 1. In (**B**), liposomes contained phosphatidylcholine and cardiolipin (7:1, mol/mol); in (**C**), liposomes were composed of mitochondrial lipids. (**D**) Rat liver mitochondria were treated with 100 μg/mg protease K. PMSF (1 mM) was then added, and mitochondria were incubated for a further 10 min. Mitochondria were recovered by centrifugation. They were incubated with 50 nM $mBax_{FL}$, in the absence (not shown) or in the presence of 5 nM tBid for 30 min at 30 °C. Mitochondria were solubilized with 2% (w/v) CHAPS and proteins fractionated on a Superdex 200 column (16/60). Fractions of 2 ml were collected, and every second fraction was analysed by SDS/PAGE and Western blotting with antibodies against Bax. Results are representative of at least two independent experiments.

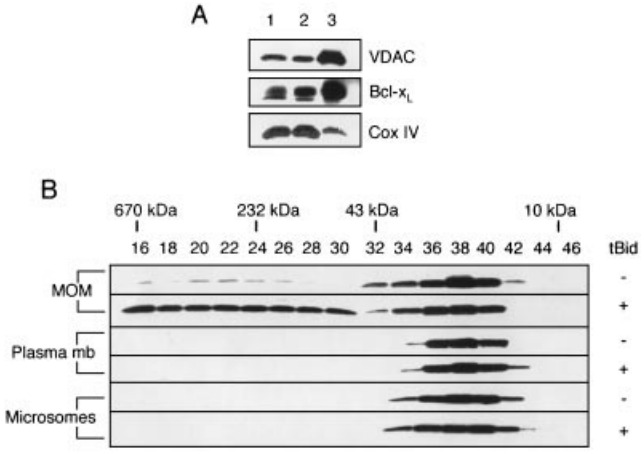

Figure 3 tBid induces oligomerization of recombinant Bax in MOM vesicles

(**A**) Portions of 50 μg of protein isolated from rat liver mitochondria (lane 1), mitoplasts (lane 2) and MOM (lane 3) were analysed by Western blotting with antibodies directed against VDAC, Bcl-X_L and cytochrome *c* oxidase subunit 4 (Cox IV). (**B**) Rat liver MOM and mitoplasts, rat erythrocyte plasma membrane (plasma mb) and rat liver microsomes were incubated with 50 nM $mBax_{FL}$, in the absence or in the presence of 5 nM tBid as indicated. Bax oligomerization was analysed by gel filtration and Western blotting as described in the legend to Figure 1. Results are representative of at least two independent experiments.

cleaved with caspase-8 in order to produce the active C-terminal fragment tBid [40]. In the presence of tBid, $mBax_{FL}$ triggered the release of cytochrome *c* from isolated mitochondria (Figure 1D). At the concentrations tested, tBid alone had no effect (Figure 1D). However, higher concentrations of tBid (2–5 nM) induced the release of cytochrome *c* in the absence of $mBax_{FL}$ (results not shown), probably through activation of Bak [21]. As shown in Figure 1(F), 5 nM tBid induced Bak oligomerization.

Since Bax permeabilizes the MOM to cytochrome *c* only when it is oligomerized (Figure 1C), we tested whether tBid was able to induce $mBax_{FL}$ insertion and oligomerization in mitochondria. Bax_{FL} insertion in mitochondrial membranes was determined by alkali treatment of membranes. In the absence of tBid, a small amount of Bax_{FL} was alkali-resistant, and therefore inserted into mitochondria (Figure 1E). In the presence of tBid, the amount of Bax_{FL} associated with the mitochondrial pellet after alkali treatment was greatly increased, showing that tBid was able to induce Bax_{FL} insertion into mitochondrial membranes (Figure 1D). Bax oligomerization was tested by gel filtration after protein extraction with CHAPS, a detergent that does not artificially induce Bax oligomerization, in contrast with Triton X-100 or octyl glucoside [29]. $mBax_{FL}$ did not oligomerize spontaneously, since it was recovered in fractions corresponding to its molecular mass (Figure 1F). In contrast, in the presence of tBid, Bax_{FL} was detected as a monomer, but also in fractions corresponding to high-molecular-mass complexes (Figure 1F) that have been shown previously to correspond, at least in part, to Bax oligomers [30]. Endogenous Bak also oligomerized in the presence of tBid (Figure 1F), confirming previous studies [37], whereas Bcl-X_L was detected in low-molecular-mass fractions even in the presence of tBid (Figure 1F).

Bax does not oligomerize in liposomes

We have shown previously that $mBax_{FL}$ does not oligomerize in liposomes [45]. Moreover, $mBax_{FL}$, in contrast with $oBax_{FL}$, did not induce the release of cytochrome *c* from liposomes, either in the absence or in the presence of tBid ([45]; Figure 2A). However, specific mitochondrial phospholipids, such as cardiolipin [46], which are absent from the liposome preparations, might be involved in Bax oligomerization. To test this hypothesis, liposomes containing 30% (w/v) cardiolipin were prepared and incubated with $mBax_{FL}$. It was found that $mBax_{FL}$ did not oligomerize in cardiolipin-containing liposomes in the presence or absence of tBid (Figure 2B). In order to reconstitute the lipid composition of mitochondrial membranes, lipids were isolated from mitochondria and reconstituted in liposomes. Again, no oligomerization was observed (Figure 2C). We concluded that a mitochondrial factor, which is not a phospholipid, might be required for tBid-induced Bax oligomerization. In support of these data, when mitochondria were pretreated with protease K, tBid failed to trigger $mBax_{FL}$ oligomerization (Figure 2D). Altogether, these results suggest that a mitochondrial protein induces Bax_{FL} oligomerization in the presence of tBid.

tBid induces Bax oligomerization in MOMs

Bax insertion seems to occur into the MOM [33,35]. To test whether the mitochondrial protein required for Bax oligomerization is also present in the MOM, this membrane was purified from mouse liver mitochondria. MOM and mitochondrial inner membrane markers, including the voltage-dependent anion channel (VDAC), Bcl-X_L and cytochrome *c* oxidase subunit 4, were analysed to monitor MOM purification. As shown in Figure 3(A), a significant enrichment of the MOM was obtained, even though some contamination of this membrane by the mitochondrial inner membrane remained. Analysis of the MOM by electron microscopy revealed that these membranes formed unilamellar vesicles (results not shown). When $mBax_{FL}$ was

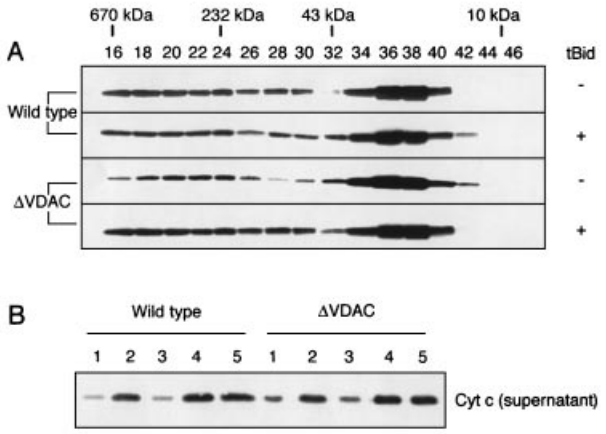

Figure 4 Oligomerization of recombinant Bax in yeast mitochondria is independent of VDAC

Mitochondria were isolated from wild-type and ΔVDAC yeast cells. (**A**) Mitochondria were incubated with 50 nM $mBax_{FL}$ in the absence or in the presence of 5 nM tBid as indicated. Bax oligomerization was analysed by gel filtration as described in the legend to Figure 1. (**B**) Mitochondria were incubated in the absence of any recombinant protein (lane 1), or with 100 nM $mBax_{FL}$ (lane 2), 1 nM tBid (lane 3), 100 nM $mBax_{FL}$ plus 1 nM tBid (lane 4) or 100 nM $oBax_{FL}$ (lane 5). After incubation for 10 min, mitochondria were recovered by centrifugation, and the presence of cytochrome *c* (Cyt c) released into the supernatant was analysed by Western blotting with a polyclonal antibody. Similar results were obtained with two independent mitochondrial preparations.

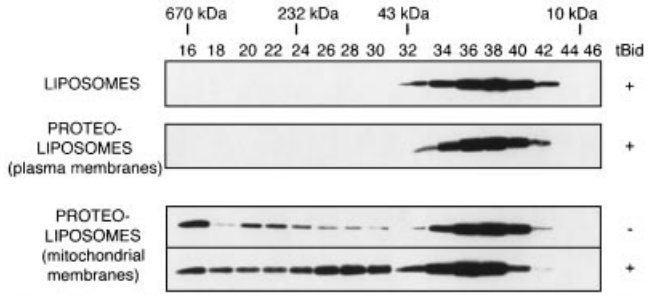

Figure 5 Reconstitution of oligomerization of recombinant Bax in proteoliposomes

Liposomes prepared from mitochondrial lipids and phosphatidylcholine were mixed with either erythrocyte plasma membrane or mitochondrial membranes solubilized with Triton X-100, or solubilization buffer containing the same amount of Triton X-100. Liposomes and proteoliposomes were formed by removing Triton X-100 as described in the Materials and methods section. The vesicles were then incubated with 50 nM $mBax_{FL}$ in the absence or in the presence of 5 nM tBid. Bax oligomerization was analysed by gel filtration and Western blotting as described in the legend to Figure 1. Similar results were obtained with four liposome and two proteoliposome preparations.

added to the MOM vesicles, the protein was detected mainly as a monomer (Figure 3B). However, when added together with tBid to the vesicles, $mBax_{FL}$ formed large complexes (Figure 3B).

In control experiments, Bax oligomerization was tested in plasma membranes and microsomes. $mBax_{FL}$ did not oligomerize in plasma membranes isolated from erythrocytes, or in microsomes isolated from rat liver cells (Figure 3B). These results suggested that there is a specific mitochondrial protein that is involved in tBid-induced $mBax_{FL}$ oligomerization, and that this protein is probably present in the MOM.

VDAC is not involved in Bax oligomerization

VDAC, a major component of the MOM, has been proposed to be directly involved in Bax-induced cytochrome *c* release [47]. Indeed, it was shown that Bax did not trigger the release of cytochrome *c* from mitochondria isolated from yeast cells that do not express VDAC. However, this result was not confirmed by Priault et al. [48]. Moreover, *in vivo* studies showed that the death function of Bax is largely independent of VDAC [49,50]. Nevertheless, we tested whether the VDAC protein may be involved in Bax oligomerization in mitochondria isolated from cells devoid of endogenous VDAC. The mitochondria were isolated from yeast cells deficient in the *POR1* gene (ΔVDAC).

When $mBax_{FL}$ was incubated with mitochondria isolated from either the parental wild-type or the ΔVDAC yeast strains, Bax oligomerized (Figure 4A). In a parallel experiment, $mBax_{FL}$ also induced the release of cytochrome *c* from both types of mitochondria (Figure 4B). Of note, in contrast with the results obtained with mitochondria from mammalian cells, with yeast mitochondria Bax oligomerization and the release of cytochrome *c* were independent of the presence of tBid. Altogether, these results show that, in yeast mitochondria, VDAC is not required either for Bax oligomerization or for Bax-induced cytochrome *c* release.

Reconstitution of Bax oligomerization in proteoliposomes

$mBax_{FL}$ did not oligomerize in the presence of tBid and mitochondria solubilized with 2% (w/v) CHAPS (results not shown). This suggested that formation of Bax oligomers in the presence of tBid requires an intact membrane structure. Furthermore, the results presented in Figure 2(D) indicated that mitochondrial proteins are essential. We therefore decided to reconstitute the activity responsible for Bax oligomerization in proteoliposomes. Mitochondrial membranes were solubilized with Triton X-100, and the solubilized proteins were reconstituted in liposomes containing mitochondrial lipids. In a control experiment, liposomes were prepared in the presence of Triton X-100, but in the absence of any proteins, or in the presence of plasma membrane proteins also solubilized with Triton X-100. In liposomes containing only mitochondrial lipids, $mBax_{FL}$ did not oligomerize either in the absence (results not shown) or in the presence (Figure 5) of tBid. This result confirmed that mitochondrial lipids are not sufficient to trigger Bax oligomerization. Moreover, since Triton X-100 alone can induce Bax oligomerization, this finding showed that all traces of detergent were removed during the preparation of liposomes. In contrast, Bax_{FL} oligomers were detected in proteoliposomes containing mitochondrial proteins. As shown in Figure 5, $mBax_{FL}$ oligomerization was detected even in the absence of tBid, but was significantly enhanced by tBid. $mBax_{FL}$ did not oligomerize in proteoliposomes containing proteins solubilized from the plasma membranes of erythrocytes, either in the absence (results not shown) or in the presence (Figure 5) of tBid. Therefore the mitochondrial protein required for Bax oligomerization can be extracted from the MOM and its activity reconstituted in proteoliposomes.

DISCUSSION

In the present paper, we have shown that oligomerization of Bax requires tBid and a mitochondrial protein whose identity is still unknown. It was observed previously that addition of recombinant Bax to liposomes led to the release of cytochrome *c* via

the formation of a Bax tetramer. However, in that study, the recombinant Bax protein lacked the final 19 amino acids (Bax ΔTM), and was denatured in the presence of urea and renatured in the presence of detergents [51]. Therefore one may suspect that the detergent could have artificially induced Bax oligomerization, or that the C-terminal domain of Bax exerts a negative effect on protein oligomerization.

We show that at least two proteins are required for Bax oligomerization: tBid and a mitochondrial protein. Bid is a BH3-domain-only protein that has been shown to be cleaved during apoptosis triggered by Fas or tumour necrosis factor [52,53]. The C-terminal part of Bid translocates to mitochondria, where it contributes to oligomerization of Bax or/and Bak [21,35,37]. This probably occurs through binding, via its BH3 domain, to Bax or Bak and/or to anti-apoptotic members of the Bcl-2 family. Indeed, a mutant of Bid in which this domain was deleted was unable to trigger Bax insertion and oligomerization [35]. It is unclear whether, in addition to Bid, other BH3-domain-only proteins could induce Bax insertion and oligomerization. Since activation of Bax and Bak appears to occur in Bid-independent models of apoptosis, e.g. staurosporine- or etoposide-induced apoptosis [22], it is likely that other BH3-domain-only proteins can also trigger Bax and Bak oligomerization.

In addition to tBid, we show here that Bax oligomerization requires at least one mitochondrial factor. What is the nature of such a factor? It has been proposed that tBid may be involved in the transport and recycling of mitochondrial phospholipids [54]. Therefore lipids may also be involved in Bax oligomerization. However, Bax did not oligomerize in liposomes made of lipids extracted from mitochondrial membranes. This could mean either that the mitochondrial factor is not a lipid or that the difference in the organization of phospholipids in mitochondrial membranes and in liposomes prevents Bax oligomerization in liposomes. Two results favour the first hypothesis. Indeed, not only was Bax oligomerization inhibited in mitochondria treated with protease K, but it could also be reconstituted in proteoliposomes. Therefore the mitochondrial factor required for Bax oligomerization is likely to be a protein. This protein is present in enriched MOM and is absent from other membranes, including the plasma membrane and microsomal membrane. Since the MOM preparation includes some contamination from inner-membrane proteins, we cannot strictly exclude the possibility that the protein required for Bax oligomerization is also present in the mitochondrial inner membrane.

Could this protein be VDAC? VDAC was suggested to be required for Bax-induced cytochrome *c* release [48]. To test the possible involvement of VDAC, we used wild-type and VDAC-deficient yeasts. As with mitochondria from mammalian cells, we found that Bax oligomerized in mitochondria from wild-type yeast and triggered the release of cytochrome *c*. A similar result was obtained with mitochondria isolated from VDAC-deficient yeast. This finding shows that, at least in yeast, Bax oligomerization does not require VDAC. A requirement of VDAC for Bax oligomerization in mammalian cells cannot be ruled out. Interestingly, in yeast, it appears that Bax can oligomerize independently of tBid. This suggests that the activity of the protein responsible for Bax oligomerization is dependent on tBid in mammalian but not in yeast cells.

It has been shown recently that one of the main functions of the BH3-domain-only proteins is to inhibit the activity of anti-apoptotic members of the Bcl-2 family [25]. Therefore, as a working hypothesis, we propose that inhibition of Bcl-X_L or of another anti-apoptotic protein of this family by tBid frees a mitochondrial protein whose identity is still unknown, allowing its interaction with Bax. This model supposes that, in normal cells, Bcl-X_L or another anti-apoptotic protein of this family would exert a tonic repression on this mitochondrial protein. In the absence of anti-apoptotic proteins of the Bcl-2 family, as is the case in yeast, this mitochondrial protein would be free to act as a Bax docking site, increasing its local concentration to a critical level, leading to oligomerization. In this regard, the function of such a protein would be reminiscent of the function of the receptors for some bacterial toxins at the surface of the plasma membrane [55].

In conclusion, we have shown that, in addition to tBid, a mitochondrial protein present in the MOM is required for Bax oligomerization. This protein, which may be either inserted into or simply associated with the MOM, has been extracted from the MOM and its activity reconstituted in proteoliposomes. This key step now allows further characterization of this crucial factor that participates in Bax activation.

We thank Monique Fornallaz-Mulhauser and Belen Sanchez for technical assistance, Dr Michael Forte for the gift of the yeast strains M3 and M22-2, and Dr Fred. Sherman for providing a polyclonal antibody directed against yeast cytochrome *c*.

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Received 24 June 2002/20 August 2002; accepted 23 August 2002
Published as BJ Immediate Publication 23 August 2002, DOI 10.1042/BJ20020972