Bid induces cytochrome *c*-impermeable Bax channels in liposomes

Xavier ROUCOU*, Tatiana ROSTOVTSEVA†, Sylvie MONTESSUIT*, Jean-Claude MARTINOU* and Bruno ANTONSSON‡[1]

*Département de Biologie Cellulaire, Sciences III, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland, †Department of Biology, University of Maryland, College Park, MD 20742, U.S.A., and ‡Serono Pharmaceutical Research Institute, 14 chemin des Aulx, CH-1228 Plan-les-Ouates, Geneva, Switzerland

Bax is a proapoptotic member of the Bcl-2 family of proteins. The Bax protein is dormant in the cytosol of normal cells and is activated upon induction of apoptosis. In apoptotic cells, Bax gets translocated to mitochondria, inserts into the outer membrane, oligomerizes and triggers the release of cytochrome *c*, possibly by channel formation. The BH3 domain-only protein Bid induces a conformational change in Bax before its insertion into the outer membrane. The mechanism by which Bid promotes Bax activation is not understood, and whether Bid is the only protein required for Bax activation is unclear. Here we report that recombinant full-length Bax (Bax_{FL}) does not form channels in lipid bilayers when purified as a monomer. In contrast, in the presence of Bid cut with caspase 8 (cut Bid), Bax forms ionic channels in liposomes and planar bilayers. This channel-forming activity requires an interaction between cut Bid and Bax, and is inhibited by Bcl-x_L. Moreover, in the absence of the putative transmembrane C-terminal domain, Bax does not form ionic channels in the presence of cut Bid. Cut Bid does not induce Bax oligomerization in liposomes and the Bax channels formed in the presence of cut Bid are not large enough to permeabilize vesicles to cytochrome *c*. In conclusion, our results suggest that monomeric Bax_{FL} can form channels only in the presence of cut Bid. Cut Bid by itself is unable to induce Bax oligomerization in lipid membranes. It is suggested that another factor that might be present in mitochondria is required for Bax oligomerization.

Key words: apoptosis, Bax, Bid, channel, mitochondria, oligomerization.

INTRODUCTION

Some apoptogenic factors such as cytochrome *c*, the apoptosis-inducing factor (AIF), Smac/Diablo and the serine protease HtrA2 are confined in the mitochondrial intermembrane space of normal cells. In stress-induced apoptosis, where the mitochondrial pathway is required for cell death to occur, these factors are released. Once in the cytosol, cytochrome *c* binds to Apaf-1 and together with dATP(ATP) induces the oligomerization of Apaf-1, leading to the recruitment of procaspase 9 and its processing into active caspase 9. This complex of cytochrome *c*, Apaf-1 and caspase 9 is commonly referred to as the Apaf-1 apoptosome [1,2]. The Apaf-1 apoptosome also recruits procaspase 3 and procaspase 7, which are activated by caspase 9 [3] and associates with the XIAP inhibitors of apoptosis [4]. Once released from the mitochondria, AIF translocates to the nucleus where it induces large-scale DNA fragmentation [5]. Smac/Diablo promotes apoptosis by removing the inhibitor of apoptosis proteins from caspases [6,7]. The serine protease HtrA2 is a Smac-like inhibitor of the inhibitor of apoptosis protein activities, with a serine protease-dependent cell-death-inducing activity [8].

The Bcl-2 family of proteins regulates the permeabilization of the outer mitochondrial membrane (OMM) during cell death. Proapoptotic members of the family such as Bax, Bak and Bid induce the release of apoptogenic factors, whereas anti-apoptotic members such as Bcl-2 or Bcl-x_L prevent their release. Two mechanisms have been proposed to explain how mitochondrial apoptogenic factors are released [9,10]. The release would occur either after the rupture of the OMM or after the formation of specific channels in the outer membrane. The channel model is based on the structural similarity between four proteins of the Bcl-2 family, namely Bcl-x_L, Bcl-2, Bid and Bax and the pore-forming domains of bacterial toxins [11–15]. Moreover, Bcl-2, Bcl-x_L, Bax and Bid form channels when incorporated into lipid membranes [16–19]. It remains to determine whether such channels are formed *in vivo* and if they indeed permeabilize the OMM to cytochrome *c*, AIF and Smac/Diablo.

Bax is a proapoptotic protein essential for apoptosis in sympathetic and motor neurons [20]. Recently, Bax was shown to form channels in lipid membranes only when present as oligomers [21]. On isolated mitochondria, Bax monomers did not trigger the release of cytochrome *c*, whereas Bax oligomers did [21]. Interestingly, in normal cells, Bax is a soluble monomeric protein present in the cytosol or loosely associated with mitochondria. After induction of apoptosis, the protein translocates to mitochondria, where it forms large oligomers that are inserted into the OMM [22–24]. Bax oligomers may be responsible for the formation of transmembrane channels large enough to transport apoptogenic factors. Indeed, Bax oligomers constituted by four molecules form a channel, which is able to permeabilize liposomes to cytochrome *c* [25].

The BH3 domain-only protein Bid triggers Bax activation. Engagement of the cell-death receptors leads to activation of pro-caspase 8 and subsequent caspase 8 cleavage of Bid into two fragments: a C-terminal fragment (t^cBid) and an N-terminal fragment (t^nBid). t^cBid then translocates to the mitochondria where it triggers Bax and Bak oligomerization [23,26,27]. Bax insertion and oligomerization into the OMM occurs after a Bid-induced conformational change in the N-terminal domain of the protein [26,28]. To gain insight into the mechanism of Bid-induced Bax activation, we have produced the full-length Bax

Abbreviations used: AIF, apoptosis-inducing factor; $Bax_{\Delta C}$, Bax with the 20-amino-acid C-terminal domain truncated; Bax_{FL}, full-length Bax; CF, 5,6-carboxyfluorescein; cut Bid, Bid cut with caspase 8; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; VDAC, voltage-dependent anion channel.

[1] To whom correspondence should be addressed (e-mail bruno.antonsson@serono.com).

(Bax_{FL}) protein in a soluble monomeric form. We report that, following its interaction with Bid cut with caspase 8 (cut Bid), Bax in the presence of liposomes does not oligomerize but forms cationic channels that are permeable to 5,6-carboxyfluorescein (CF) but not cytochrome *c*. On the contrary, we confirm that oligomeric Bax forms channels large enough to permeabilize liposomes to cytochrome *c*. Therefore Bid alone is not able to induce the formation of cytochrome *c*-permeable Bax channels in artificial lipid membranes.

MATERIALS AND METHODS

Protein purification

Bax_{FL} with a tag of six histidines at the N-terminus was expressed in the pBAD plasmid in *Escherichia coli* [29]. Monomeric Bax was recovered in the soluble bacterial fraction and purified by chromatography on nickel-nitrilotriacetic 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.

Monomeric Bax with the 20-amino-acid C-terminal domain truncated ($Bax_{\Delta C}$), oligomeric Bax_{FL} and $Bax_{\Delta C}$ were purified as described previously [18]. Caspase-8 cut wild-type mouse Bid (cut Bid) and its mutants Bid mIII-2 ($I^{93}GDE^{96} \rightarrow AAAA$) and mIII-3 ($G^{94} \rightarrow A$) were obtained from purified full-length Bid as described previously [30]. Bcl-x_L and Bcl-x_Lm ($G^{138} \rightarrow A$) were produced as described previously [28].

Liposome-channel activity assay

CF containing liposomes were prepared as described in [21] but with a modified lipid composition. Briefly, 0.5 mg of phosphatidylcholine and 0.5 mg of phosphatidylglycerol were dried under nitrogen and solubilized in 1 ml PBS, pH 7.2, containing 20 mM CF and 30 mg of octyl glucoside/ml. Incubation was carried out for 3 h at 20 °C. Liposomes were then isolated after filtering through a Sephadex G-25 column (1.5 cm × 20 cm) and dialysed overnight against PBS at 4 °C. Liposomes were diluted to give a suitable fluorescence measurement. Recombinant proteins were added as indicated in the Figures, and the change in fluorescence was recorded as a function of time with excitation at 488 nm and emission at 520 nm.

For cytochrome *c* efflux, liposomes were obtained by sonication/extrusion. Briefly, dried lipids were hydrated in 1 ml of PBS, pH 7.2, containing 5 mg of cytochrome *c* and sonicated for 5 × 30 s (400 W). Vesicles were sized using 200 nm polycarbonate filters and separated from unincorporated cytochrome *c* by a 100000 ***g*** centrifugation for 30 min at 4 °C (Kontron Instruments; rotor TFT 80.4). Recombinant proteins were added as indicated in the Figures. After incubation for 10 min, liposomes were centrifuged for 30 min at 4 °C and 100000 ***g***, and the supernatant analysed by Western blotting with anti-cytochrome *c* antibodies.

Gel-filtration analysis

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

Electrophysiological recordings

Bilayer membranes were formed from monolayers made from a 1 % (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, U.S.A.) in hexane on 70–80 μm diameter orifices in the 15-μm-thick Teflon partition that separated the two chambers [31,32]. The membranes were made in asymmetrical solutions: 200 mM KCl (*cis* side) and 50 mM KCl (*trans* side), each containing 1 mM $MgCl_2$, 5 mM Hepes, pH 7.0. The membrane potential was applied using Ag/AgCl electrodes in 3 M KCl, 15 % (w/v) agarose bridges. The current was amplified by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.) in the voltage clamp mode. Data were filtered by a low-pass eight-pole Butterworth filter (Model 9002; Frequency Devices, Haverhill, MA, U.S.A.) at 15 kHz, recorded simultaneously on a chart recorder and directly saved into the computer memory with a sampling frequency of 50 kHz. Amplitude analysis was performed by software developed in-house. The membrane chamber and headstage were isolated from the external-noise sources with double high-μ metal screen (Amuneal Manufacturing Corp., Philadelphia, PA, U.S.A.). All measurements were made at 23 ± 2 °C.

RESULTS

Bid induces a Bax-channel activity in CF-charged liposomes

Due to the poor solubility of Bax_{FL}, most *in vitro* studies have been performed with recombinant Bax with the hydrophobic C-terminal domain truncated. However, we reasoned that the C-terminal domain, which enhances targeting and insertion to mitochondria *in vivo*, might be required for Bid to trigger Bax insertion into membranes. Therefore we have produced the monomeric Bax_{FL}. Moreover, in order to simulate the activation of Bid by caspase 8, as it occurs in Fas-mediated apoptosis, Bid was proteolytically modified by cutting with caspase 8 [12,13]. This cleavage produced a 15.5 kDa C-terminally-active fragment and a 6.5 kDa N-terminal fragment [30].

When added to liposomes, oligomeric Bax_{FL} permeabilized the vesicles to CF (Figure 1A), confirming previous studies [21]. In the absence of cut Bid, monomeric Bax_{FL} did not trigger the release of CF (Figure 1A), even at a concentration of 0.5 μM (results not shown). When cut Bid was added together with Bax in the assay, an increase in fluorescence was detected, indicating the permeabilization of the liposomes. Cut Bid itself is able to permeabilize liposomes at a concentration above 100 nM [30]. This effect involved the destabilization of lipid bilayers. However, at the concentration tested in our experiment, cut Bid did not induce the release of CF (Figure 1A).

The BH3 domain of Bid is essential for binding to Bax via its BH1 domain [33]. To determine whether induction of channel-forming activity requires an interaction between the two proteins, two mutants in the BH3 domain of Bid were tested: Bid mIII-2 ($^{93}IGDE^{96} \rightarrow AAAA$) and Bid mIII-3 ($G^{94} \rightarrow A$). Both mutants were less efficient in inducing the release of CF (Figure 1B), suggesting that Bid interaction with Bax through its BH3 domain was required to induce Bax-channel activity. This result also confirmed that cut Bid by itself was not responsible for the release of CF from liposomes, since Bid mIII-2 and Bid mIII-3 were shown to have the same activity as the wild-type protein in destabilizing liposomes at high concentration [30].

Bcl-x_L was able to prevent the release of CF (Figure 1C). To determine whether this inhibition required Bcl-x_L binding to Bax, a mutant of Bcl-x_L that is able to interact with Bid but not with Bax was tested. In this mutant, Gly^{138} was replaced by an alanine (Bcl-x_Lm: $G^{138} \rightarrow A$) [11,28]. Bcl-x_Lm did not prevent the release

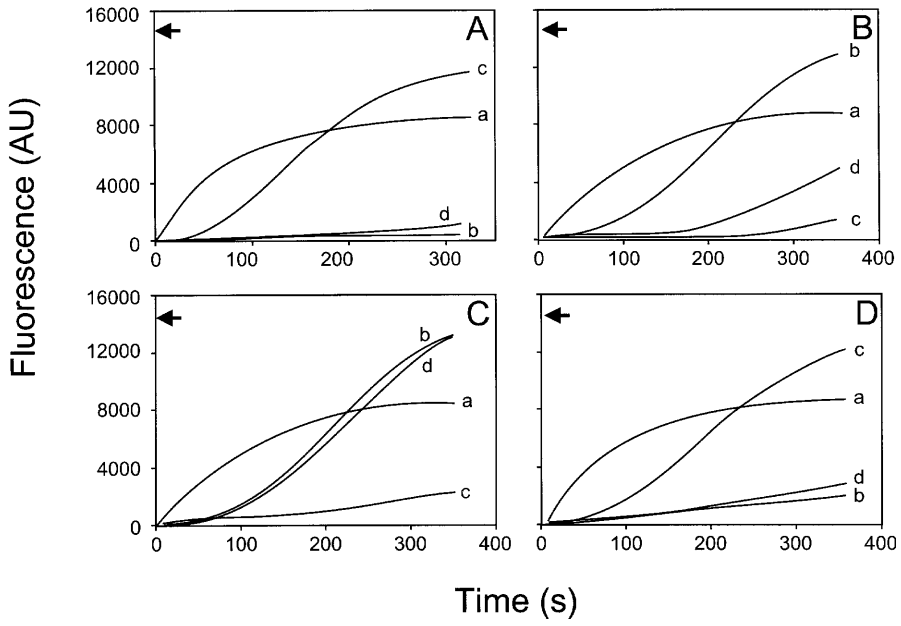

Figure 1 Bid-induced Bax-channel activity in CF-charged liposomes

Liposomes containing 20 mM CF were incubated at room temperature in PBS (pH 7.5). Proteins were added at 100 nM (Bax_{FL}, Bcl-x_L) or 16 nM (cut Bid) and fluorescence change was measured over time. In (**A**, **B**, **C**, **D**), liposomes were incubated with recombinant oligomeric Bax_{FL} (a). In (**A**), liposomes were incubated with monomeric Bax_{FL} (b), monomeric Bax_{FL} and cut Bid (c) or cut Bid (d). In (**B**), liposomes were incubated with monomeric Bax_{FL} and cut Bid wild type (b), cut Bid mIII-2 (c), cut Bid mIII-3 (d). In (**C**), liposomes were incubated with monomeric Bax_{FL} and cut Bid, in the absence (b) or in the presence of Bcl-x_L wild type (c) or Bcl-x_Lm (d). In (**D**), liposomes were incubated with monomeric Bax deleted from the C-terminal 24 residues ($Bax_{\Delta C}$) (b), cut Bid and monomeric Bax_{FL} (c), cut Bid and $Bax_{\Delta C}$ (d).

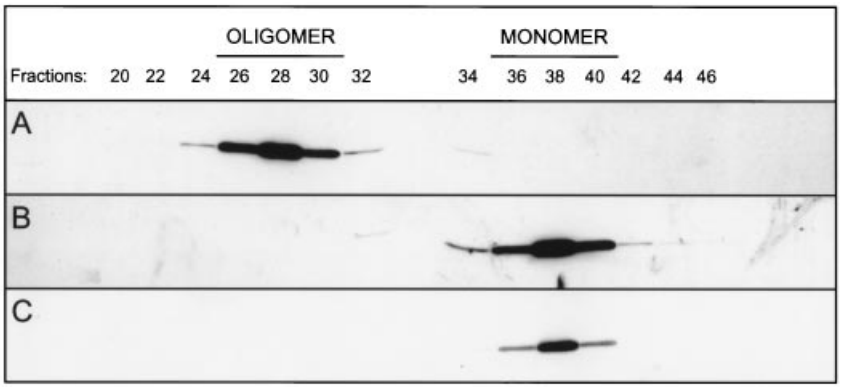

Figure 2 Gel-filtration analysis of Bax

The column was run at a flow rate of 1 ml/min, and fractions of 2 ml were collected. From each fraction, 30 μl aliquots were analysed by Western blotting. A 50 μg portion of recombinant oligomeric (**A**) or monomeric (**B**) Bax_{FL} was separated by gel filtration. (**C**), liposomes were incubated with 500 nM monomeric Bax_{FL} and 80 nM cut Bid. After centrifugation at 100000 ***g***, liposomes were solubilized with 2% (w/v) CHAPS and Bax_{FL} analysed by gel filtration.

of CF from liposomes (Figure 1C). Thus Bcl-x_L prevented cut Bid-induced Bax-channel-forming activity through its interaction with Bax and not with Bid.

Bax contains a hydrophobic domain at its C-terminus, which is thought to mediate the insertion of the protein into the OMM. We reasoned that if Bax formed a channel in liposomes, its insertion into the lipid bilayer via the C-terminal domain might be essential. To test this hypothesis, a monomeric Bax mutant $Bax_{\Delta C}$ was assayed. In the presence of cut Bid, $Bax_{\Delta C}$ was unable to induce the release of CF (Figure 1D). In contrast, oligomeric $Bax_{\Delta C}$ permeabilized the liposomes as described in [21]. We concluded that cut Bid-induced Bax-channel-forming activity required the C-terminal domain of the Bax protein.

Bax oligomerization is required for channel-forming activity in liposomes [21]. We therefore assayed for Bax oligomerization status by gel filtration. When incubated with liposomes, oligomeric Bax_{FL} was detected in fractions 20–24, whereas monomeric Bax_{FL} was detected in fractions 36–40 (Figures 2A and 2B, respectively). When monomeric Bax_{FL} was incubated in the presence of cut Bid and liposomes, Bax was still detected in fractions 36–40 (Figure 2C). Addition of cardiolipin, a lipid found preferably in the mitochondrial membranes, in the lipo-

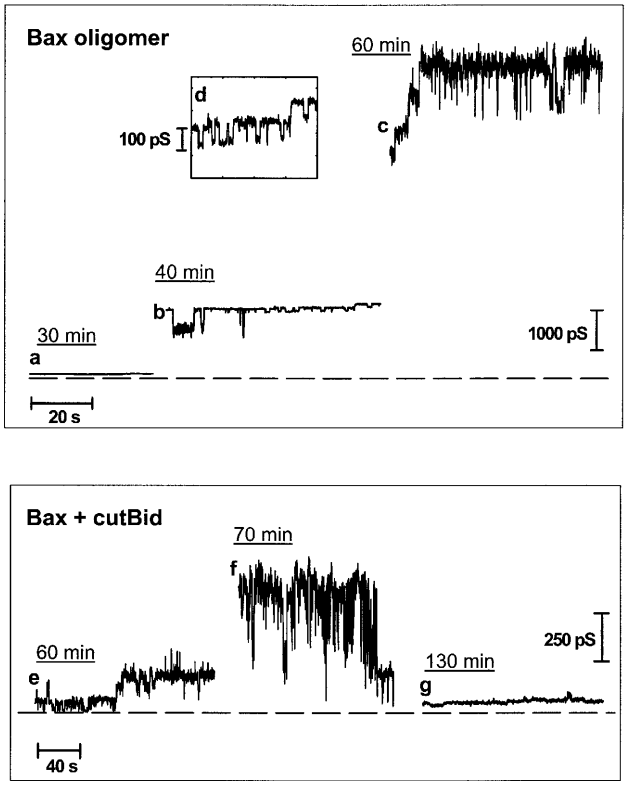

Figure 3 Bax monomer channel in the presence of cut Bid is different from Bax oligomer channels

Continuous current recordings of channel formation in planar membranes after addition of 40 nM Bax_{FL} oligomer (**a**–**c**) or 20 nM monomeric Bax_{FL}/2 nM cut Bid (**e**–**g**) to the aqueous solution, which consisted of 200 mM KCl in the *cis* side and 50 mM KCl in the *trans* side, 1 mM $MgCl_2$ and 5 mM Hepes, pH 7.0. (**d**) The inset shows the current trace in (**b**) on a larger scale. The time after addition of the recombinant proteins is indicated. The broken line indicates the zero-current level. The applied voltage was clamped at +20 mV and the time resolution was 30 ms. Note that the scale in the top panel is four times larger than in the bottom panel.

somes did not promote Bax oligomerization (results not shown). Therefore, under conditions in which cut Bid induces a Bax channel-forming activity on liposomes, Bax remains in a monomeric form.

Cut Bid-induced monomeric Bax channel is different from oligomeric Bax channel

In the presence of cut Bid, monomeric Bax_{FL} could permeabilize liposomes to CF by two mechanisms: either by forming channels or by destabilizing the lipid membrane. To distinguish between these two possibilities, Bax-channel activity was tested in planar-lipid bilayers. Addition of oligomeric Bax_{FL} produced channels that consistently appeared within 10–15 min with gradual increase in conductance from 90 to 1800 and 5400 pS (Figures 3a–3d). Often, after reaching the high total conductance level of 10–20 nS the membranes ruptured. The decrease in the lifetime of the membranes in the presence of Bax proteins has been described previously [34]. In our experiments, membranes lasted for as long as 1 h after the formation of the first channels.

The channels were non-selective, as the reversal potential was zero in a 4-fold gradient of KCl salt (Figure 4A). This result confirmed the ability of oligomeric Bax_{FL} to form large channels, as observed in previous studies in which Bax lacking the C-terminal hydrophobic domain was used [18,35]. In contrast, monomeric Bax_{FL} did not increase the conductance of the bilayers at the concentration tested (up to 80 nM; results not shown). However, in the presence of cut Bid, Bax_{FL} monomer formed channels with rather low conductances, which fluctuated during the experiment. In the experiment illustrated in Figures 3(e)–3(g), conductance varied between 60 and 90 pS (traces e and g) and 650 pS (trace f). Recordings could frequently continue for several hours without rupture of the membrane. It was shown that cut Bid on its own could destabilize artificial bilayers at concentrations higher than 100 nM [30]. In our experiments, we never used concentrations of cut Bid higher than 6 nM, and in control experiments concentrations up to 50 nM did not show any effect on the membrane conductance or stability (results not shown).

Channels formed by oligomeric Bax_{FL} and monomeric Bax_{FL} in the presence of cut Bid displayed different properties. Oligomeric Bax_{FL} channels were non-selective, whereas channels formed by monomeric Bax_{FL} in the presence of cut Bid demonstrated high cation selectivity (Figure 4B). Moreover, both oligomers and monomers of Bax_{FL} induced channels with 100–200 pS conductance in the presence of cut Bid (Figure 4C). However, only Bax_{FL} oligomers formed a significant amount of large 1000–1500 pS channels (Figure 4C). Still larger conductance steps in the range 4000–8000 pS were formed by oligomeric Bax_{FL}, but these were rather rare events and are not shown in the histogram for clarity of illustration.

Cut Bid-induced monomeric Bax channel does not transport cytochrome *c*

Bax-dependent transport of cytochrome *c* in liposomes requires the formation of pores constituted by at least four Bax molecules [25]. Since cut Bid was unable to induce Bax oligomerization in liposomes, we tested whether cut Bid-induced Bax-channel-forming activity could permeabilize liposomes to cytochrome *c*. We prepared liposomes containing cytochrome *c*. Addition of oligomeric Bax_{FL} induced the release of cytochrome *c* (Figure 5), in accordance with previous results obtained with Bax truncated from the 19 C-terminal residues [25]. In contrast, monomeric Bax_{FL} was unable to permeabilize liposomes to cytochrome *c*, either in the absence or in the presence of cut Bid. We concluded that in contrast with oligomeric Bax, the channels formed by monomeric Bax in the presence of cut Bid allows the transport of low-molecular-mass compounds, like CF, but does not form a pore large enough to permeabilize liposomes to cytochrome *c*.

DISCUSSION

To prevent the artificial formation of Bax oligomers [21,29,36], Bax was purified in the absence of any detergent. The molecular mass of Bax_{FL} was ascertained to correspond to the calculated monomeric molecular mass by gel filtration (result not shown). In the present study, we describe a new Bid-induced Bax_{FL}-channel-forming activity. In the absence of cut Bid, recombinant monomeric Bax_{FL} was unable to induce the release of CF from vesicles and did not induce any conductance in planar-lipid bilayers. Bax-channel-forming activity in liposomes or in planar-lipid bilayers was observed only in the presence of cut Bid. This channel-forming activity required a physical contact between the Bax and Bid proteins, since mutants in the BH3 domain of Bid that have been shown to be unable to interact with Bax did not activate the Bax channel. Moreover, by preventing the interaction between cut Bid and Bax, Bcl-x_L abrogated the channel formation.

Bid was previously shown to induce a conformational change of Bax, preceding its insertion and oligomerization in the OMM [26,28]. The folded conformation of monomeric Bax_{FL} is highly

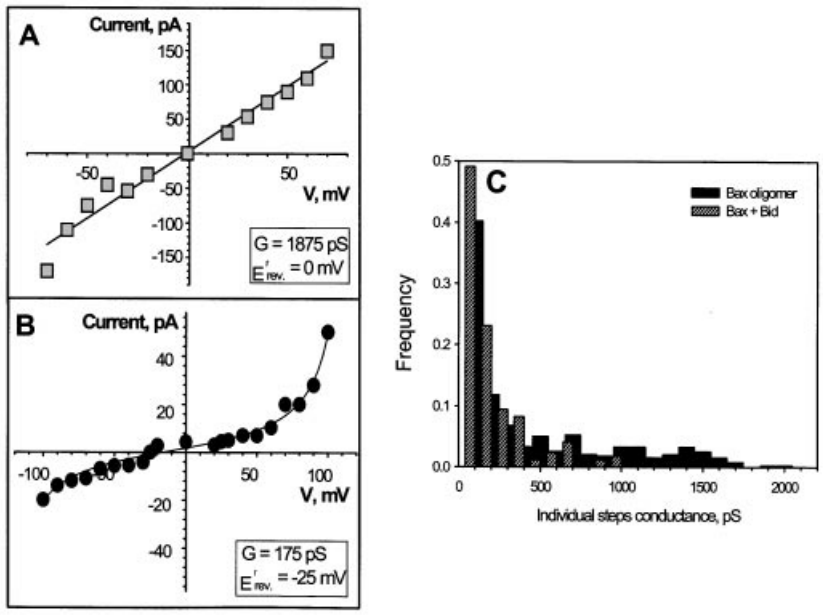

Figure 4 Current–voltage plots and distribution of channel conductances formed by Bax oligomer and Bax monomer in the presence of cut Bid

(**A**) Oligomeric Bax_{FL} forms large unselective channels. The current–voltage dependences were obtained for the total conductance induced by 40 nM oligomeric Bax_{FL} in the *cis* side. (**B**) Monomeric Bax_{FL} activated by cut Bid forms smaller cation-selective channels. The current–voltage dependences were obtained for the total conductance induced by 60 nM cut Bid-activated monomeric-Bax_{FL} in the *cis* and *trans* sides. (**C**) Distribution of individual conductance steps produced by oligomeric Bax_{FL} and monomeric Bax_{FL} activated by cut Bid. Data were compiled from four and three separate experiments with oligomeric Bax_{FL} (10–44 nM) and cut Bid-activated monomeric Bax_{FL} (30–60 nM), respectively. The conductance values were taken for all applied voltages. Only distinct vertical current increments were measured.

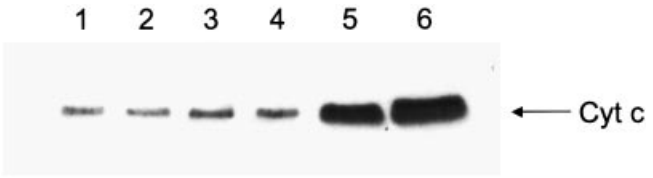

Figure 5 Release of cytochrome *c* from liposomes is mediated by Bax oligomers

Vesicles were prepared with 5 mg of horse cytochrome *c*/ml. Vesicles were incubated without any protein (**1**), with 100 nM Bax_{FL} monomer (**2**), 10 nM cut Bid (**3**), 100 nM Bax_{FL} monomer and 10 nM cut Bid (**4**), 100 nM Bax_{FL} oligomer (**5**), or with 0.1 % (w/v) Triton X-100 to release all the cytochrome *c* content (**6**). After incubation for 10 min, vesicles were centrifuged and cytochrome *c* detected in the supernatant by Western blotting with a polyclonal anti-cytochrome *c* antibody.

compact and the C-terminus is buried within a hydrophobic core [14]. This compact structure might prevent the protein from inserting into and permeabilizing membranes. Elucidation of the structure of Bax revealed that the C-terminal α9 helix occupies a hydrophobic pocket in the protein proposed to bind the BH3 domain of other Bcl-2 family members, and therefore, to mediate the heterodimer formation [14]. Since the C-terminal domain enhances Bax association with mitochondria during apoptosis [22,37], one may propose that Bid induces the dislocation of helix α9, which then inserts into the membrane. Based on energy requirements to disengage helix α9 from the hydrophobic pocket, the suggestion that a BH3 domain of another Bcl-2 family member could be responsible for the exposure of that helix was not favoured by Suzuki et al. [14]. However, we observed in the present study that cut Bid is not able to induce a Bax-channel activity when Bax has helix α9 truncated. Recombinant monomeric $Bax_{\Delta C}$ could not permeabilize vesicles. Therefore we propose that cut Bid may trigger the exposure of the C-terminal domain of Bax, now allowing its insertion into membranes and the formation of channels. Phospholipids may also contribute together with cut Bid to the exposure of helix α9 of the Bax protein and its insertion.

Gel-filtration analysis revealed that in the presence of cut Bid, Bax remained as a monomer. It is therefore unlikely that the Bax-channel-forming activity measured in the presence of cut Bid was due to the formation of Bax/Bax homo-oligomers or Bax/cut Bid hetero-oligomers. However, an association between Bax/Bax or Bax/cut Bid cannot be excluded, since such interactions might be disrupted under conditions in which gel-filtration experiments were carried out.

The cut Bid-induced monomer-Bax_{FL} channel displayed different properties compared with the oligomeric Bax_{FL} channel. First, it has a smaller conductance and it is cation-selective, whereas the Bax oligomer channel has a large conductance and is unselective. Secondly, the channel is non-permeable to cytochrome *c*, in accordance with the finding that only Bax oligomers are able to permeabilize membranes to cytochrome *c* [21,25]. The impact of the formation of such a channel *in vivo* would depend on Bax localization. In the OMM, the channel might modulate the activity of the voltage-dependent anion channel (VDAC). Interestingly, Bcl-x_L was shown to promote the open configuration of VDAC [38], and the authors proposed that, in contrast, proapoptotic proteins such as Bax might directly or indirectly induce VDAC closure. On the other hand, an interaction between Bax and the adenine nucleotide translocator in the inner mitochondrial membrane (IMM) was shown in co-immunoprecipitation and yeast two-hybrid experiments [39], suggesting that Bax may be present in the IMM under some

circumstances. Some authors have reported that Bax induces the opening of the permeability transition pore when added to isolated mitochondria, by a mechanism that is undefined [40,41]. By forming small cation channels in the IMM, Bax may induce a transient depolarization, thus activating the voltage-dependent permeability transition pore. The consequence of the formation of low-conductance Bid-induced Bax channels in the OMM is unclear.

Since Bid does not induce Bax oligomerization in liposomes, whereas it does in isolated mitochondria [26], our results suggest that Bax oligomerization might occur specifically in mitochondrial membranes and might require mitochondrial components.

T.R. thanks Sergey Bezrukov for helpful discussions.

REFERENCES

1 Zou, H., Li, Y., Liu, X. and Wang, X. (1999) An APAF-1 cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. **274**, 11 549–11 556

2 Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R. and Alnemri, E. S. (1999) Cytochrome *c* and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J. Biol. Chem. **274**, 17 941–17 945

3 Cain, K., Brown, D. G., Langlais, C. and Cohen, G. M. (1999) Caspase activation involves the formation of the apoptosome, a large (700 kDa) caspase-activating complex. J. Biol. Chem. **274**, 22 686–22 692

4 Bratton, S. W., Walker, G., Srinivasula, S. M., Sun, X.-M., Alnemri, E. S. and Cohen, G. M. (2001) Recruitment, activation and retention of caspase-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. EMBO J. **20**, 998–1009

5 Suzin, S. A., Lorenzo, H. K., Zamzani, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature (London) **397**, 441–446

6 Verhagen, A. M., Ekert, P. G., Pakush, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J. and Vaux, D. L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell **102**, 43–53

7 Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell **102**, 33–42

8 Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi, R. (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol. Cell **8**, 613–621

9 Desagher, S. and Martinou, J.-C. (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol. **10**, 369–377

10 Martinou, J.-C. and Green, D. R. (2001) Breaking the mitochondrial barrier. Nature Rev. Mol. Cell Biol. **2**, 63–67

11 Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L. et al. (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature (London) **381**, 335–341

12 Chou, J. J., Li, H., Salvesen, G. S., Yuan, J. and Wagner, G. (1999) Solution structure of BID, an intracellular amplifier of apoptotic signaling. Cell **96**, 615–624

13 McDonnell, J. M., Fushman, D., Milliman, C. L., Korsmeyer, S. J. and Cowburn, D. (1999) Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. Cell **96**, 625–634

14 Suzuki, M., Youle, R. Y. and Tjandra, N. (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell **103**, 645–654

15 Petros, A. M., Medek, A., Nettesheim, D. G., Kim, D. H., Yoon, H. S., Swift, K., Matayoshi, E. D., Oltersdorf, T. and Fesik, S. W. (2001) Solution structure of the antiapoptotic protein bcl-2. Proc. Natl. Acad. Sci. U.S.A. **98**, 3012–3017

16 Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M. and Reed, J. C. (1997) Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U.S.A. **94**, 5113–5118

17 Minn, A. J., Velez, P., Schendell, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M. and Thompson, C. B. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature (London) **385**, 353–357

18 Antonsson, B., Conti, F., Ciavatta, A. M., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.-J., Mazzei, G. et al. (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science **277**, 370–372

19 Schendel, S. L., Azimov, R., Pawlowski, K., Godzik, A., Kagan, B. L. and Reed, J. C. (1999) Ion channel activity of the BH3 only Bcl-2 family member, BID. J. Biol. Chem. **274**, 21 932–21 936

20 Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, E. M., Snider, W. D. and Korsmeyer, S. J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron **17**, 401–411

21 Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J. C. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome *c* release from mitochondria. Biochem. J. **345**, 271–278

22 Wolter, K. G., Hsu, Y.-T., Smith, C. L., Nechustan, A., Xi, X.-G. and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. **139**, 1281–1292

23 Antonsson, B., Montessuit, S., Sanchez, B. and Martinou, J.-C. (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J. Biol. Chem. **276**, 11 615–11 623

24 Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A. and Saikumar, P. (2001) Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. **276**, 18 361–18 374

25 Saito, M., Korsmeyer, S. J. and Schlesinger, P. H. (2000) BAX-dependent transport of cytochrome *c* reconstituted in pure liposomes. Nat. Cell Biol. **2**, 553–555

26 Eskes, R., Desagher, S., Antonsson, B. and Martinou, J.-C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol. Cell Biol. **20**, 929–935

27 Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B. and Korsmeyer, S. J. (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome *c*. Genes Dev. **14**, 2060–2071

28 Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J.-C. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. J. Cell Biol. **144**, 891–901

29 Montessuit, S., Mazzei, G., Magnenat, E. and Antonsson, B. (1999) Expression and purification of full-length human Bax alpha. Protein Expr. Purif. **15**, 202–206

30 Kudla, G., Montessuit, S., Eskes, R., Berrier, C., Martinou, J.-C., Ghazi, A. and Antonsson, B. (2000) The destabilization of lipid membranes induced by the C-terminal fragment of caspase 8-cleaved bid is inhibited by the N-terminal fragment. J. Biol. Chem. **275**, 22 713–22 718

31 Montal, M. and Mueller, P. (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc. Natl. Acad. Sci. U.S.A. **69**, 3561–3566

32 Rostovtseva, T. K. and Bezrukov, S. M. (1998) ATP transport through a single mitochondrial channel, VDAC, studied by current fluctuation analysis. Biophys. J. **74**, 2365–2373

33 Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L. and Korsmeyer, S. J. (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. **10**, 2859–2869

34 Basanez, G., Nechustan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K. A., Hsu, Y.-T., Zimmerberg, J. and Youle, R. J. (1999) Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. U.S.A. **96**, 5492–5497

35 Schlesinger, P. H., Gross, A., Yin, X. M., Yamamoto, K., Saito, M., Waksman, G. and Korsmeyer, S. J. (1997) Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc. Natl. Acad. Sci. U.S.A. **94**, 11 357–11 362

36 Hsu, Y.-T. and Youle, R. J. (1997) Nonionic detergents induce dimerization among members of the Bcl-2 family. J. Biol. Chem. **272**, 13 829–13 834

37 Nechushtan, A., Smith, C. L., Hsu, Y. T. and Youle, R. J. (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J. **18**, 2330–2341

38 Vander Heiden, M. G., Li, X. X., Gottlieb, E., Hill, R. B., Thompson, C. B. and Colombini, M. (2001) Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. J. Biol. Chem. **276**, 19 414–19 419

39 Marzo, I., Brenner, C., Zamzani, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L. A., Prevost, M.-C., Xie, Z., Matsuyama, S., Reed, J. C. and Kroemer, G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science **281**, 2027–2031

40 Narita, M., Simizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H. and Tsujimoto, Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. Proc. Natl. Acad. Sci. U.S.A. **95**, 14 681–14 686

41 Pastorino, J. G., Tafani, M., Rothman, R. J., Marcineviciute, A., Hoek, J. B. and Farber, J. L. (1999) Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. J. Biol. Chem. **274**, 31 734–31 739

Received 23 November 2001/22 January 2002; accepted 12 February 2002