Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome *c* release from mitochondria

Bruno ANTONSSON[1], Sylvie MONTESSUIT, Sandra LAUPER, Robert ESKES and Jean-Claude MARTINOU
Serono Pharmaceutical Research Institute, Ares-Serono International S. A., 14, chemin des Aulx, CH-1228 Plan-les Ouates, Geneva, Switzerland

Bax is a Bcl-2-family protein with pro-apoptotic activity that can form channels in lipid membranes. The protein has been shown to trigger cytochrome *c* release from mitochondria both *in vitro* and *in vivo*. Recombinant human Bax isolated in the presence of detergent was found to be present as an oligomer with an apparent molecular mass of approx. 160000 Da on gel filtration. When Bax was isolated in the absence of detergent the purified protein was monomeric with an apparent molecular mass of 22000 Da. Bax oligomers formed channels in liposomes and triggered cytochrome *c* release from isolated mitochondria, whereas monomeric Bax was inactive in both respects. Incubation of the monomeric Bax with 2% octyl glucoside induced formation of oligomers that displayed channel-forming activity in liposomes and triggered cytochrome *c* release from mitochondria. Triton X-100, Nonidet P-40 and *n*-dedecyl maltoside also activated monomeric Bax, whereas CHAPS had no activating effect. In cytosolic extracts from mouse liver, Bax migrated at a molecular mass of 24000 Da on gel filtration, whereas after incubation of the cytosol with 2% octyl glucoside Bax migrated at approximately 140000 Da. These results show that oligomeric Bax possesses channel-forming activity whereas monomeric Bax has no such activity.

Key words: apoptosis, Bcl-2 protein, cell death.

INTRODUCTION

Apoptosis or programmed cell death is an essential physiological process required for a normal development and for maintenance of tissue homoeostasis [1]. The Bcl-2 family of proteins are central regulators of the intracellular apoptotic signalling cascades [2]. These proteins can be subdivided into two groups, one with anti-apoptotic activity (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1) and one with pro-apoptotic activity (Bax, Bak, Bok/Mtd, Bcl-Xs, Bid, Bad, Bik/Nbk, Bim, Blk) [3–5]. The proteins contain four conserved amino acid regions, referred to as the Bcl-2 homology domains (BH1, BH2, BH3 and BH4). The activity of these proteins appears to be regulated, at least partly, by formation of homo- and hetero-complexes [6–9]. In the pro-apoptotic proteins Bax and Bak, the BH3 domain is essential for complex formation as well as for their 'killing' effect [10–14]. Several of these proteins also contain a hydrophobic C-terminal domain thought to be responsible for targeting the proteins to intracellular membranes [8,15].

Structural studies of the monomeric soluble form of Bcl-X_L, one of the anti-apoptotic family members, showed that the protein is composed of two hydrophobic central helices (α5 and α6), which are surrounded by five amphipathic helices [16]. The BH1, BH2 and BH3 domains are located in close proximity on the surface of the protein and form a hydrophobic cleft. A peptide of the BH3 domain from the pro-apoptotic protein Bak has been shown to bind to this hydrophobic cleft [17]. The solution structure of Bcl-X_L is reminiscent of diphtheria toxin and the colicins A and E1. These toxins contain a pore-forming domain which functions as a membrane channel that allows passage of ions or small polypeptides. It was subsequently shown that the anti-apoptotic Bcl-X_L and Bcl-2 as well as the pro-apoptotic Bax possess channel-forming activity, although the channel-forming requirements and the properties of the channels differ between the anti- and pro-apoptotic proteins [18–21]. Bcl-X_L and Bcl-2 form channels only at low pH and these channels are cation-selective, whereas Bax can form channels at neutral pH that are anion-selective.

Mitochondria have been shown to have a crucial function in apoptosis [22,23]. Changes in the mitochondrial permeability, release of mitochondrial proteins and a decrease in the mitochondrial membrane potential are early events during apoptosis. One of the mitochondrial proteins released into the cytosol is cytochrome *c*, which has been shown to form a complex with Apaf-1, dATP and pro-caspase 9 [24]. Upon complex formation the caspase is activated and triggers the downstream activation of other caspases, ultimately leading to cell death. It has been shown that overexpression or activation of Bax in cells, as well as addition of purified recombinant Bax to isolated mitochondria, can trigger the release of cytochrome *c* [25–27]. However, the molecular mechanism by which Bax triggers cytochrome *c* release has not been determined. Here, we show that monomeric recombinant Bax cannot form channels in liposomes, nor trigger cytochrome *c* release from isolated mitochondria. In contrast, oligomeric Bax is capable of forming channels and triggers cytochrome *c* release.

MATERIAL AND METHODS

Materials

The Superdex 200 (16/60), Mono Q, and Q-Sepharose columns were from Pharmacia (Uppsala, Sweden) and the 4–20% gradient polyacrylamide gels were from Novex (San Diego, CA, U.S.A.). Octyl glucoside (OG) was from Alexis (San Diego, CA, U.S.A.), Nonidet P-40 (NP-40), CHAPS and *n*-dodecyl maltoside were from Boehringer Mannheim (Mannheim, Germany) and Triton X-100 was from Fluka (Buchs, Switzerland). Polyclonal anti-Bax antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and monoclonal anti-Hsp70 antibodies were from Affinity Bioreagents (Golden, CO, U.S.A.). The polyclonal anti-cytochrome *c* antibodies were raised in-house.

Abbreviations used: DTT, dithiothreitol; OG, octyl glucoside; NP-40, Nonidet P-40; Ni-NTA, Ni^{2+}-nitrilotriacetate.

[1] To whom correspondence should be addressed (e-mail bruno.antonsson.ch-gva03@serono.com).

Purification of oligomeric C-terminal truncated Bax ($Bax_{\Delta C}$)

$Bax_{\Delta C}$ was expressed in the pET23D plasmid in *Escherichia coli* with a tag of six histidines at the N-terminus. The bacterial cell paste was suspended in 100 mM Hepes/NaOH/300 mM NaCl/2 mM $MgCl_2$/5 mM 2-mercaptoethanol/1 mM PMSF/1 mM benzamidine/1 % Triton X-100/10 μg/ml antipain/10 μg/ml soya bean trypsin inhibitor/2 μl of each pepstatin A, aprotinin, α1-antitrypsin and leupeptin, pH 8.0, and broken by two passages through a French press cell at a cell pressure of 110000 kPa. The protein was recovered in the soluble fraction after centrifugation at 35000 ***g*** for 30 min and purified by chromatography on Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose. The soluble bacteria extract was applied to the column equilibrated with 50 mM Hepes/NaOH/300 mM NaCl/5 mM 2-mercaptoethanol/10 % glycerol/30 mM imidazole, pH 8.0, and the protein was eluted with a linear 30–200 mM imidazole gradient. The $Bax_{\Delta C}$ pool from the Ni column was purified further on a DEAE–Sepharose column equilibrated with 25 mM Tris/HCl/5 mM 2-mercaptoethanol/50 mM NaCl, pH 7.5, and developed with a linear 50–500 mM NaCl gradient. The purified protein was concentrated, dialysed against 25 mM Hepes/NaOH/0.2 mM dithiothreitol (DTT)/30 % (v/v) glycerol, pH 7.5, and stored at −80 °C.

Purification of oligomeric full-length Bax

Full-length Bax was purified as described previously [28]. In summary, Bax was expressed in *E. coli* with a tag of six histidines at the N-terminus. The protein was recovered in the soluble fraction in the presence of 1 % Triton X-100 and purified on Ni-NTA–agarose followed by anion-exchange chromatography on a Q-Sepharose column. The purified protein was dialysed against 25 mM Hepes/NaOH/0.2 mM DTT/1 % (w/v) OG/30 % glycerol, pH 7.5, and stored at −80 °C.

Purification of monomeric $Bax_{\Delta C}$

$Bax_{\Delta C}$ was expressed in the pET23D plasmid in *E. coli* with a tag of six histidines at the N-terminus. The bacterial cell paste was suspended in 100 mM Hepes/NaOH/300 mM NaCl/2 mM $MgCl_2$/5 mM 2-mercaptoethanol/1 mM PMSF/1 mM benzamidine/10 μg/ml antipain/10 μg/ml soya bean trypsin inhibitor/2 μg/ml each of pepstatin A, aprotinin, α1-antitrypsin and leupeptin, pH 7.2, and broken by two passages through a French press cell at a cell pressure of 110000 kPa. $Bax_{\Delta C}$ was recovered in the soluble fraction after centrifugation at 35000 ***g*** for 30 min and purified on Ni-NTA–agarose. The sample was loaded on a column equilibrated with 50 mM Hepes/NaOH/300 mM NaCl/5 mM 2-mercaptoethanol/10 % glycerol/30 mM imidazole, pH 7.2, and $Bax_{\Delta C}$ was eluted with a linear gradient of 30–200 mM imidazole. $Bax_{\Delta C}$ was further purified on a MonoQ column equilibrated with 25 mM Tris/HCl/5 mM 2-mercaptoethanol/50 mM NaCl, pH 8.0, and developed with a linear 50–1000 mM NaCl gradient. The purified protein was concentrated, dialysed against 25 mM Hepes/NaOH/0.2 mM DTT/30 % (v/v) glycerol, pH 7.5, and stored at −80 °C.

Incubation of $Bax_{\Delta C}$ with detergent

Monomeric $Bax_{\Delta C}$ at a concentration of 100 μM was incubated in 25 mM Hepes/NaOH/0.2 mM DTT/10 % (v/v) glycerol, pH 7.5, with detergents as indicated in the Figures at 4 °C for 30 min or 1 h. After incubation the samples were diluted in the same buffer without detergent to obtain the concentrations suitable for further analysis.

Gel-filtration analysis

Gel filtrations were performed at 4 °C on a Superdex 200 (16/60) column equilibrated with 25 mM Hepes/NaOH/300 mM NaCl/0.2 mM DTT, pH 7.5, with or without 2 % (w/v) OG at a flow rate of 1 ml/min. The column was calibrated with gel-filtration standard proteins from Pharmacia giving the following elution volumes: thyroglobulin, 669000 Da, 53.5 ml; ferritin, 440000 Da, 60.7 ml; catalase, 232000 Da, 70.5 ml; aldolase, 158000 Da, 72.0 ml; BSA, 67000 Da, 80.9 ml; ovalbumin, 43000 Da, 86.6 ml; and chymotrypsinogen A, 25000 Da, 95.5 ml. A 500-μl sample was loaded on to the column and the eluate was monitored at 280 nm. Fractions of 2 ml were collected and analysed by dot immunoblotting.

Dot immunoblotting

Each fraction (20 μl) from the gel-filtration column was spotted on a PVDF membrane, dried and blocked in PBS containing 2 % skimmed-milk powder and 0.1 % Tween 20. After blocking, the membrane was incubated with the anti-Bax antibodies, washed, incubated with the peroxidase-labelled antibody and developed with ECL* from Amersham.

Liposome-channel activity assay

5,6-Carboxyfluorescein-containing liposomes were prepared as described earlier [20]. Briefly, 400 μg of phosphatidylcholine, 400 μg of phosphatidylserine and 230 μg of cholesterol were solubilized in PBS containing 20 mM carboxyfluorescein and 30 mg/ml OG by incubation at room temperature for 3 h. The liposomes were isolated subsequently by passage over a Sephadex G-25 column (1.5 × 20 cm). The column was equilibrated with PBS and the upper third loaded with 20 mM carboxyfluorescein in PBS; a 1-ml sample was then applied and the liposomes were eluted at a flow rate of 0.1 ml/min. The liposomes were dialysed against PBS and diluted to a final volume of 8 ml. For the channel activity assay, 20–50 μl of the liposomes were diluted in 1 ml of PBS to give a suitable fluorescence measurement. Increasing concentrations of Bax or control proteins, as indicated in the Figures, were added and the change in fluorescence was recorded over time.

Mitochondrial cytochrome *c* release assay

The assay was performed as described in [26]. Mitochondria were isolated from mouse liver on a Percol gradient and suspended in 10 mM Hepes/NaOH/210 mM mannitol/70 mM sucrose/1 mM EDTA, pH 7.4, at a concentration of 80 absorbance units/ml, measured at 600 nm. The final mitochondrial concentration in the assay was 3 absorbance units/ml in a total reaction volume of 200 μl. The incubations were performed at 30 °C for 30 min in 15 mM Hepes/NaOH/125 mM KCl/4 mM $MgCl_2$/5 mM KH_2PO_4/0.5 mM EGTA/5 mM succinate/5μM rotenone, pH 7.4, with addition of proteins as indicated in Figure 5 (see below). At the end of the incubation period the samples were centrifuged at 10000 ***g*** for 3 min. The supernatant was removed and analysed by Western blotting for cytochrome *c*. The mitochondrial pellet was analysed for cytochrome *c* and Bax. Hsp70 was used as an equal-loading control for the pellet fraction.

RESULTS

Gel-filtration analysis on Superdex 200

Recombinant human Bax α protein truncated of the 20 amino acids hydrophobic domain at the C-terminal ($Bax_{\Delta C}$) was purified from *E. coli* cells disrupted in buffer containing 1 % Triton X-

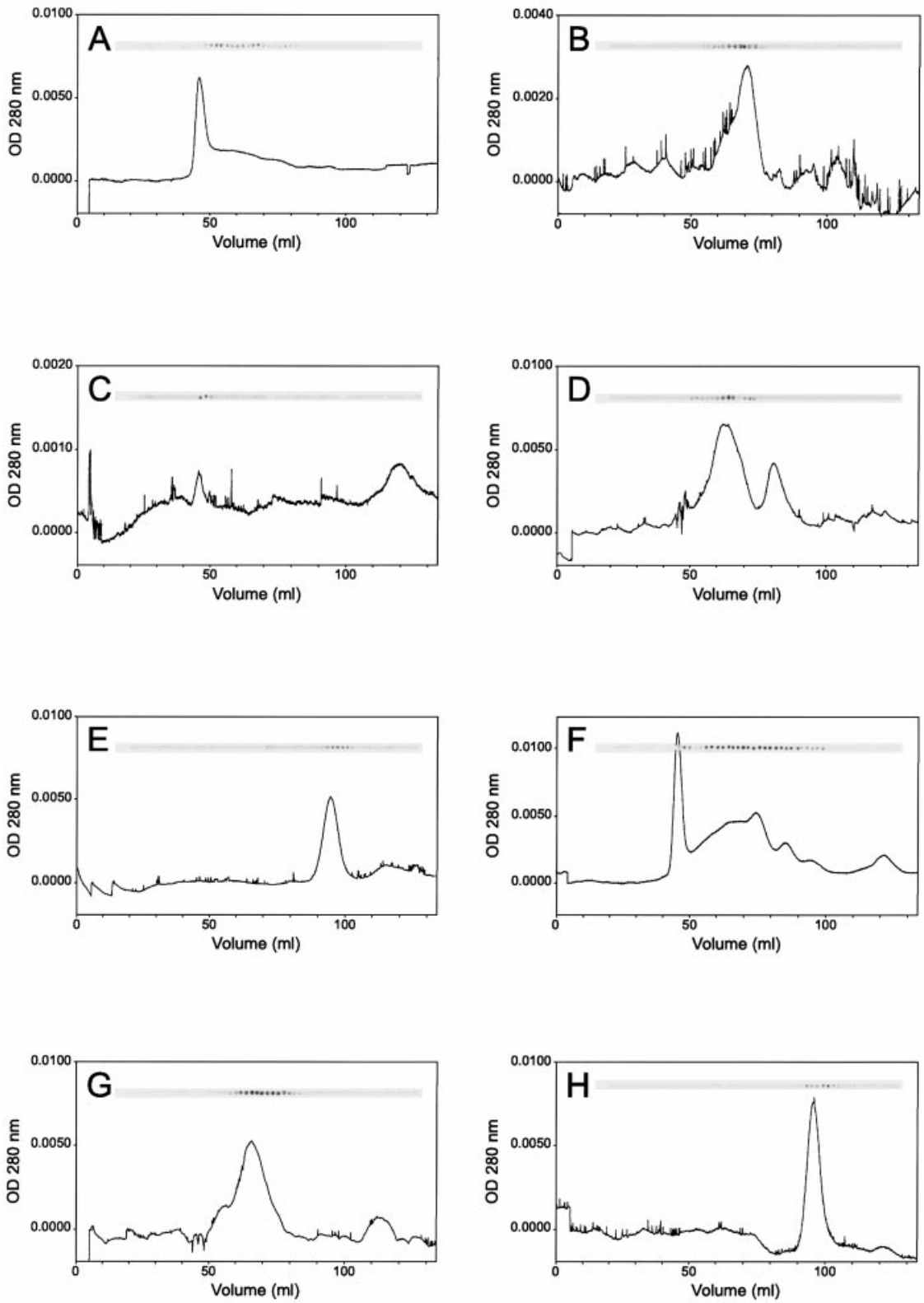

Figure 1 Gel-filtration analysis of Bax on Superdex 200

The column was equilibrated with 25 mM Hepes/NaOH/300 mM NaCl/0.2 mM DTT, pH 7.5, with or without 2% OG as indicated below. The column was run at a flow rate of 1 ml/min and 50 μg of the proteins were loaded in 500 μl. The eluate was monitored at 280 nm and fractions of 2 ml were collected. From each fraction 20 μl was spotted on to a PVDF membrane and analysed with anti-Bax antibodies. The dot immunoblots are inserted above the chromatograms. (**A**) Oligomeric $Bax_{\Delta C}$ without OG. (**B**) Oligomeric $Bax_{\Delta C}$ with 2% OG. (**C**) Full-length Bax without OG. (**D**) Full-length Bax with 2% OG. (**E**) Monomeric $Bax_{\Delta C}$ without OG. (**F**) Monomeric $Bax_{\Delta C}$, pre-incubated with 2% OG for 1 h at 4 °C, analysed in the absence of OG. (**G**) Monomeric $Bax_{\Delta C}$, pre-incubated with 2% OG for 1 h at 4 °C, analysed in the presence of 2% OG. (**H**) Monomeric $Bax_{\Delta C}$ pre-incubated with 2% CHAPS for 1 h at 4 °C, analysed in the absence of detergent.

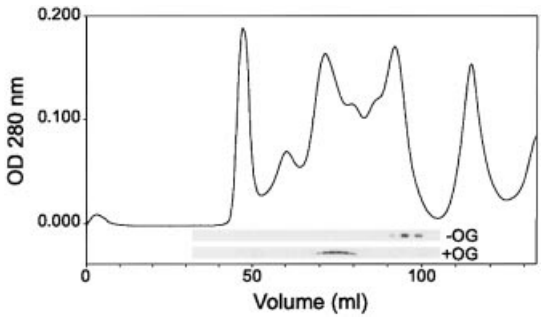

Figure 2 Gel-filtration analysis of mouse liver cytosolic extract on Superdex 200

The column was equilibrated with 25 mM Hepes/NaOH/300 mM NaCl/0.2 mM DTT, pH 7.5, with or without 2% OG. The column was run at a flow rate of 1 ml/min, the eluate was monitored at 280 nm and fractions of 2 ml were collected. Cytosol extract (500 μl) containing 1 mg of total protein was analysed on the column in the absence of OG. Every second fraction was analysed by Western blotting with anti-Bax antibodies. In a second run 500 μl of cytosol extract was pre-incubated with 2% OG for 1 h at 4 °C and analysed on the column in the presence of 2% OG. The Western blots with anti-Bax antibodies of the eluted fractions are shown below the chromatogram (−OG and +OG).

100. This $Bax_{\Delta C}$ protein migrated as a mixture of oligomer complexes with molecular masses ranging from greater than 500000 Da to approx. 160000 Da on the column in the absence of detergent in the migration buffer (Figure 1A). In the presence of 2% OG in the migration buffer $Bax_{\Delta C}$ migrated as an oligomer of approx. 160000 Da (Figure 1B). Full-length Bax isolated in the presence of detergent migrated in the absence of detergent as an oligomer complex larger than 500000 Da and in the presence of 2% OG in the migration buffer as an oligomer of 250000 Da (Figures 1C and 1D). However, $Bax_{\Delta C}$ isolated in the absence of detergent migrated at a molecular mass of 22000 Da (Figure 1E). The calculated monomeric molecular mass of $Bax_{\Delta C}$ is 19960 Da. When monomeric $Bax_{\Delta C}$ was pre-incubated with 2% OG and analysed subsequently in the presence of 2% OG the protein migrated with a molecular mass of 160000 (Figure 1G). In the absence of detergent $Bax_{\Delta C}$ pre-incubated with OG migrated as a mixture of oligomer complexes with molecular masses up to and above 500000 Da (Figure 1F). The elution profile of the OG-pre-incubated monomeric $Bax_{\Delta C}$ was similar to $Bax_{\Delta C}$ isolated in the presence of detergent (Figures 1A and 1B). In contrast, monomeric $Bax_{\Delta C}$ pre-incubated with 2% CHAPS and analysed in buffer without detergent migrated at 22000 Da as a monomer (Figure 1H).

In mouse liver extract Bax is found in the cytosol. When liver cytosol was analysed by gel filtration in the absence of detergent, Bax migrated as a 24000-Da protein (Figure 2, −OG). After pre-incubation of the cytosol with 2% OG, Bax migrated with a molecular mass of 140000 in the presence of 2% OG in the migration buffer (Figure 2, +OG). In the absence of detergent in the migration buffer, Bax in the cytosol pre-incubated with OG migrated as an oligomer complex larger than 500000 Da (results not shown). The presence of 2% OG in the sample and migration buffers did not change the elution profile monitored at 280 nm significantly.

Bax channel-forming activity in carboxyfluorescein-charged liposomes

Oligomeric $Bax_{\Delta C}$ triggered carboxyfluorescein release from the liposomes in a concentration-dependent manner (Figure 3A). Full-length Bax was present as an oligomer on gel-filtration chromatography and it induced carboxyfluorescein release in a similar manner to oligomeric $Bax_{\Delta C}$ (Figure 3B). All dilutions of the full-length Bax were adjusted with dialysis buffer to keep the OG concentration (0.0004%) constant at all Bax concentrations, including the control. In contrast, monomeric $Bax_{\Delta C}$ (Figure 1E) showed no channel-forming activity in the liposomes (Figure 3C). No channel activity was detected, even at a $Bax_{\Delta C}$ concentration of 500 nM, which is more than 80-fold higher than the concentration required (6 nM) for channel-forming activity with oligomeric $Bax_{\Delta C}$. However, when monomeric $Bax_{\Delta C}$ was pre-incubated with 2% OG for 30 min at 4 °C the protein oligomerized and showed channel-forming activity (Figure 3D). Monomeric $Bax_{\Delta C}$ was incubated with 2% OG at a concentration of 100 μM and then diluted in buffer without detergent to give final $Bax_{\Delta C}$ concentrations of between 1.5 and 12.5 nM in the assay. All dilutions were adjusted with buffer containing 2% OG to keep the OG concentration constant at 0.00025% at all Bax concentrations assayed. As seen in Figure 3(D), OG at this concentration in the absence of Bax had no effect on carboxyfluorescein release. The release of carboxyfluorescein increased with increasing $Bax_{\Delta C}$ concentration similar to that seen with $Bax_{\Delta C}$ isolated as an oligomer. However, it could not be excluded that the combination of Bax and detergent could have a destabilizing effect on the liposomes, triggering carboxyfluorescein release. To exclude this possibility, liposomes were incubated in assay buffer with 0.02% OG, the fluorescence was recorded for 2 min and then monomeric $Bax_{\Delta C}$ was added and the recording continued for another 4 min. At 0.02% OG, addition of the detergent induced an immediate pulse release of carboxyfluorescein; thereafter the fluorescence level remained constant (Figure 3E). Addition of monomeric $Bax_{\Delta C}$ at a concentration of 5 or 100 nM did not induce additional carboxyfluorescein efflux. When 100 nM $Bax_{\Delta C}$ was added 2 min prior to 0.02% OG the same result was obtained (Figure 3E). At the addition of the detergent an immediate increase in fluorescence was observed but no Bax channel activity was detected. Bcl-2 and Bcl-X_L (100 nM) did not form channels under the assay conditions used (Figure 3F). The two proteins were pre-incubated with 2% OG as described above for monomeric $Bax_{\Delta C}$, and subsequently diluted and assayed (100 nM protein, 0.003% OG) for channel-forming activity. Pre-incubation with OG did not induce channel-forming activity (Figure 3F). Taken together, these results show that the channel-forming activity detected after pre-incubation of monomeric $Bax_{\Delta C}$ with OG is an intrinsic property of the $Bax_{\Delta C}$ oligomer and not an effect of the detergent.

The data in Figure 3(E) suggested that low OG concentrations did not induce channel-forming activity in monomeric $Bax_{\Delta C}$. To test the OG concentration-dependence, monomeric $Bax_{\Delta C}$ was incubated for 1 h at 4 °C with increasing OG concentrations between 0 and 3.5% (w/v). The protein was incubated at a concentration of 100 μM and diluted in buffer without detergent to give a final $Bax_{\Delta C}$ concentration of 5 nM in the assay. Incubation with 0.5% OG did not induce any channel-forming activity; 1% OG partially activated the protein and optimal channel-forming activity was obtained with 2% OG. Further increase in the OG concentration (up to 3.5%) did not increase the Bax channel-forming activity (Figure 4A). The kinetics showed that the activation is a relatively fast process; when incubated with 2% OG, monomeric $Bax_{\Delta C}$ had gained full channel-forming activity within 15 min. Indeed, after 30 s of incubation the protein had gained more than 50% of its channel-forming activity (Figure 4B). In contrast, in the presence of 0.5% OG no channel-forming activity was detected, even after a 24-h incubation at 4 °C or a 1-h incubation at 30 °C (results not

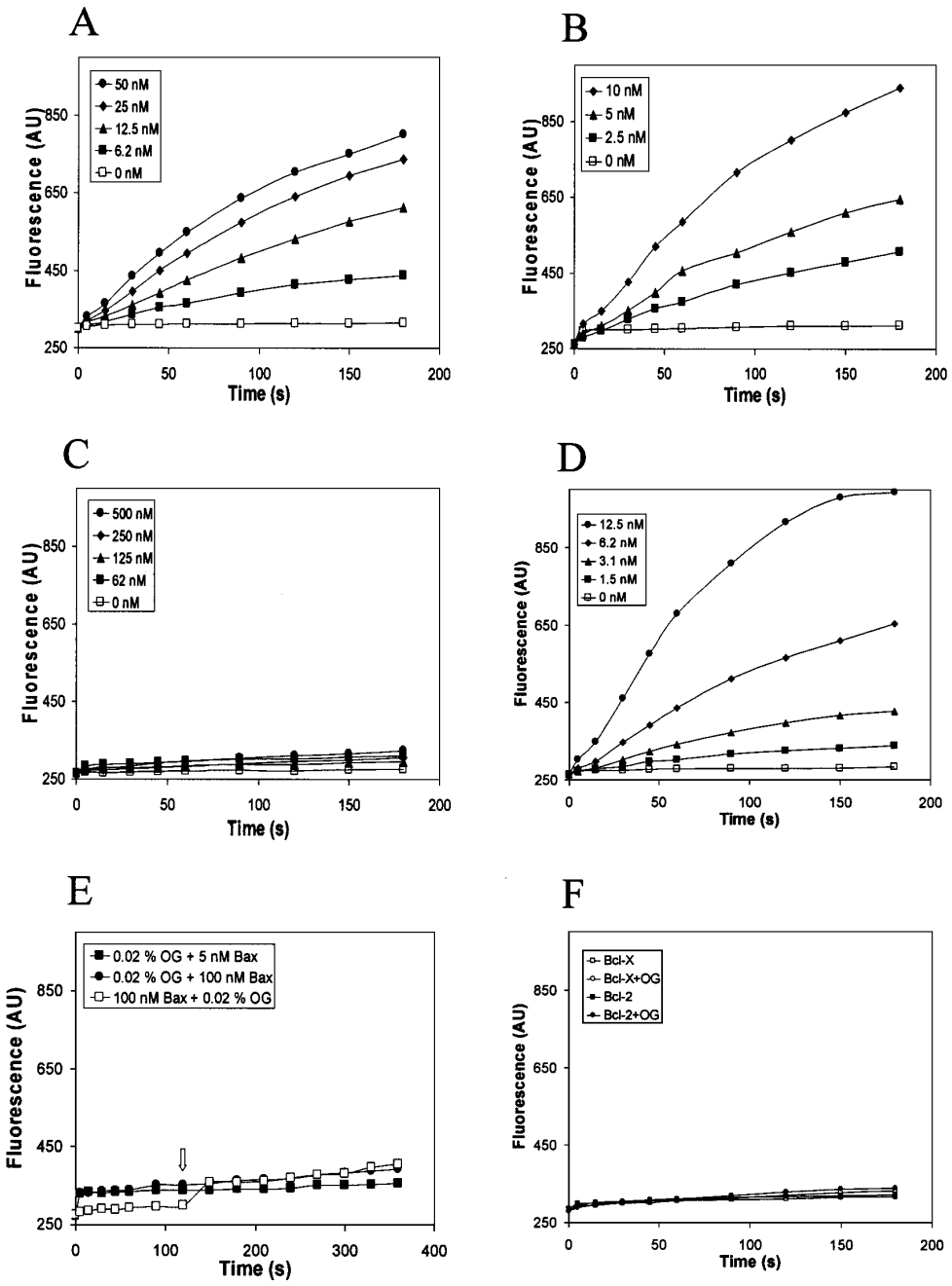

Figure 3 Bax channel-forming activity in carboxyfluorescein-charged liposomes

Liposomes containing 20 mM carboxyfluorescein were incubated at room temperature in 1 ml PBS (pH 7.5). At time zero the proteins were added at the concentrations indicated and the fluorescence was measured with excitation at 488 nm and emission at 520 nm. (**A**) $Bax_{\Delta C}$ oligomer. (**B**) Full-length Bax present as an oligomer. (**C**) Monomeric $Bax_{\Delta C}$. (**D**) Monomeric $Bax_{\Delta C}$ after pre-incubation with 2% OG for 1 h at 4 °C. All samples including the buffer control contained the same final OG concentration (0.00025%). (**E**) Monomeric $Bax_{\Delta C}$ assayed in the presence of OG without pre-incubation. OG at a final concentration of 0.02% was added to the liposomes and after 2 min of incubation 5 (■) or 100 (●) nM monomeric $Bax_{\Delta C}$ was added (arrow) and the samples were incubated for another 4 min. In addition, 100 nM monomeric $Bax_{\Delta C}$ (□) was first added and after 2 min of incubation OG (arrow) was added to a final concentration of 0.02%. (**F**) Bcl-2 and Bcl-X_L. The proteins were incubated with or without 2% OG for 1 h at 4 °C, diluted in buffer without detergent and assayed at a final concentration of 100 nM. The samples incubated in the presence of OG had a final OG concentration in the assay of 0.003%.

shown). Monomeric $Bax_{\Delta C}$ at a concentration of 100 μM was incubated for 1 h at 4 °C with 2% (w/v) OG or CHAPS and 1% (w/v) Triton X-100, NP-40 or *n*-dodecyl maltoside. Before the assay, the samples were diluted in buffer without detergent to give a final $Bax_{\Delta C}$ concentration of 5 nM in the assay. The detergent concentrations in the assay were 0.0001% for OG and CHAPS and 0.00005% for Triton X-100, NP-40 and *n*-dodecyl maltoside. At these concentrations the detergents alone did not induce any detectable release of carboxyfluorescein over background (results not shown). OG and Triton X-100 were most efficient in inducing channel-forming activity of $Bax_{\Delta C}$. NP-40 was slightly less effective the OG and Triton X-100. *n*-Dodecyl maltoside partially

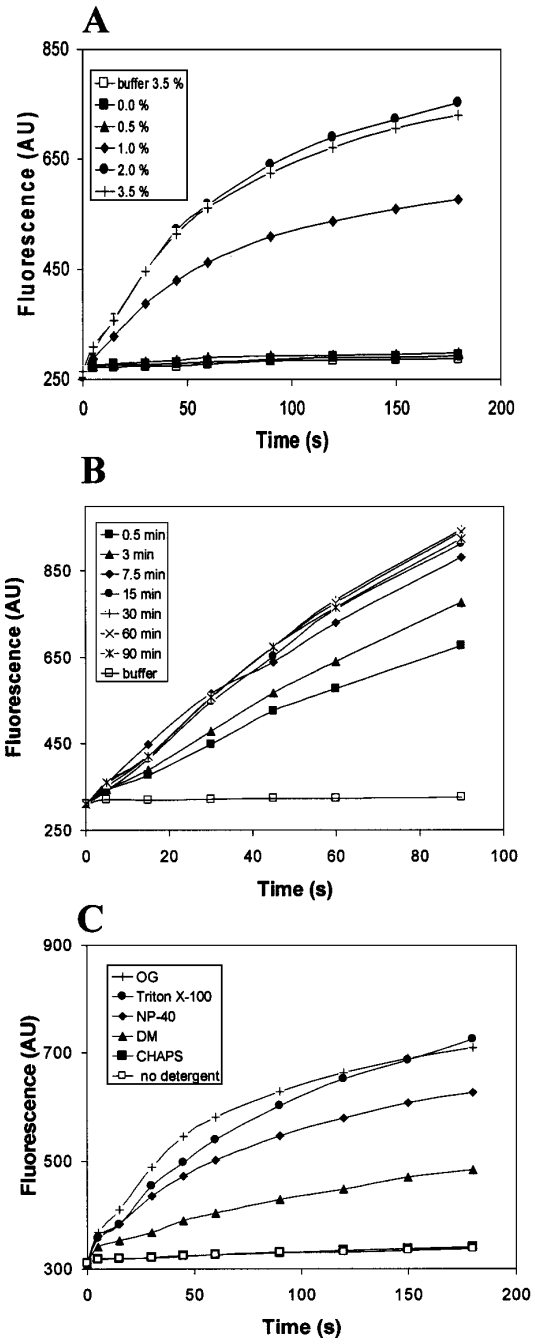

Figure 4 Detergent-induced channel-forming activity of monomeric $Bax_{\Delta C}$

(**A**) Monomeric $Bax_{\Delta C}$ (100 μM) was incubated with increasing OG concentrations, as indicated in the Figure, for 1 h at 4 °C. At the end of the incubation the protein was diluted in buffer without detergent to give a final Bax concentration of 5 nM in the assay. (**B**) Kinetics of induction of channel-forming activity. Monomeric $Bax_{\Delta C}$ (100 μM) was incubated with 2% OG and samples were withdrawn and assayed at different time points, as indicated. Immediately before assaying the samples were diluted in buffer without detergent to give a final Bax concentration of 5 nM in the assay. The final OG concentration in the assay was 0.0001%. (**C**) Channel-forming activity induced with different detergents. $Bax_{\Delta C}$ at 100 μM was incubated with 2% OG, 2% CHAPS, 1% Triton X-100, 1% *n*-dodecyl maltoside (DM) and 1% NP-40 for 1 h at 4 °C. At the end of the incubation the samples were diluted in buffer without detergent to give a final Bax concentration of 5 nM in the assay. The detergent concentrations in the assay were 0.0001% for OG and CHAPS and 0.00005% for Triton X-100, *n*-dodecyl maltoside and NP-40.

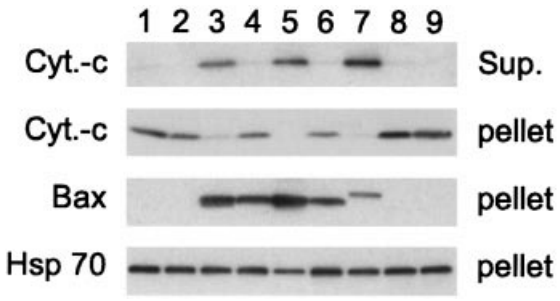

Figure 5 Bax triggered cytochrome *c* release from isolated mitochondria

Mitochondria at 3 absorbance units/ml (measured at 600 nm) were incubated at 30 °C for 30 min in 200 μl of 15 mM Hepes/NaOH/125 mM KCl/4 mM $MgCl_2$/5 mM KH_2PO_4/0.5 mM EGTA/5 mM succinate/5 μM rotenone, pH 7.4. Lane 1, buffer; lane 2, buffer with 0.09% OG; lane 3, 5 μM $Bax_{\Delta C}$ oligomer; lane 4, 5 μM $Bax_{\Delta C}$ monomer; lane 5, 5 μM $Bax_{\Delta C}$ monomer pre-incubated with 2% OG (0.09% OG in the assay); lane 6, 5 μM $Bax_{\Delta C}$ monomer without pre-incubation assayed in the presence of 0.09% OG; lane 7, 0.2 μM full-length Bax oligomer; lane 8, 5 μM Bcl-X_L; and lane 9, 5 μM Bcl-X_L pre-incubated with 2% OG (0.09% in the assay). At the end of the incubation period the samples were centrifuged at 10000 ***g*** for 3 min. The supernatant (Sup.) was removed and analysed by Western blotting for cytochrome *c* (Cyt.-c). The mitochondria pellet was analysed for cytochrome *c* and Bax. Hsp70 was used as an equal-loading control for the pellet fraction.

activated monomeric $Bax_{\Delta C}$; the channel-forming activity reached 40–50% of that obtained after activation with OG or Triton X-100. CHAPS, which failed to induce oligomerization, did not induce any channel-forming activity in monomeric $Bax_{\Delta C}$ (Figure 4C). Incubation of oligomeric $Bax_{\Delta C}$ with the detergents did not significantly alter their channel-forming activities, except in the cases of OG and Triton X-100, which appeared to increase the Bax channel-forming activity by 20–30% (results not shown).

Bax induced mitochondrial cytochrome *c* release

To investigate further the channel-forming activity of the various Bax preparations the proteins were incubated with mouse liver mitochondria and the release of cytochrome *c* was monitored by Western blotting (Figure 5). The proteins were used at a final concentration of 5 μM, except for full-length Bax, which was used at 0.2 μM. Untreated mitochondria contained cytochrome *c* and no leakage to the supernatant was detected after 30 min of incubation at 30 °C (Figure 5, lane 1). Addition of buffer containing 2% OG diluted to give the same final concentration (0.09%) as used in the protein samples did not induce any cytochrome *c* release (Figure 5, lane 2). $Bax_{\Delta C}$ isolated as an oligomer (Figure 5, lane 3) induced cytochrome *c* release into the supernatant. The same result was obtained with full-length Bax (Figure 5, lane 7). In contrast, monomeric $Bax_{\Delta C}$ (Figure 5, lane 4) did not induce cytochrome *c* release. However, monomeric $Bax_{\Delta C}$ pre-incubated with 2% OG for 1 h at 4 °C (Figure 5, lane 5) and shown to be present as an oligomer by gel-filtration chromatography was as efficient in triggering cytochrome *c* release as the preparations isolated as Bax oligomers (Figure 5, lanes 3 and 7). Monomeric $Bax_{\Delta C}$, without pre-incubation with detergent, assayed in the presence of 0.09% OG (Figure 5, lane 6) did not induce cytochrome *c* release. As expected, Bcl-X_L did not trigger cytochrome *c* release (Figure 5, lane 8) and pre-incubation with 2% OG did not activate the protein (Figure 5, lane 9). Analysis of the mitochondrial pellet fraction with anti-Bax antibodies showed that Bax was attached to the mitochondria in all conditions after addition of recombinant Bax. However, the samples containing Bax oligomer (Figure 5, lanes 3 and 5)

showed at least twice the amount of Bax attached to the mitochondria compared with the monomeric Bax samples (Figure 5, lanes 4 and 6). Comparison with the full-length Bax (Figure 5, lane 7) is difficult since 25-fold less Bax protein was added to this sample.

DISCUSSION

A possible mechanism of action for the Bcl-2-family proteins came from the three-dimensional structure of Bcl-X_L that shows similarities to the pore-forming domain of some bacterial toxins. It has since been shown that not only the anti-apoptotic proteins Bcl-2 and Bcl-X_L but also the pro-apoptotic protein Bax can form channels in lipid membranes [20,21]. It has also been demonstrated that Bax can trigger the release of cytochrome *c* from mitochondria both *in vitro* and *in vivo* [25–27]. However, the mechanism by which Bax is activated and the active structure of Bax still remain to be elucidated.

Bax is found in the cytosol of most normal healthy tissues, which indicates that the protein is kept in an inactive form in the cells. In apoptotic cells or when Bax is overexpressed it is targeted to the mitochondrial membranes, where the protein triggers cytochrome *c* release, leading to apoptosis [25,26,29–31]. The mechanism underlying the translocation has not been elucidated. Here we have shown that recombinant monomeric Bax has no channel-forming activity, nor does it trigger cytochrome *c* release from isolated mitochondria. We confirm published observations by Hsu and Youle that Bax is monomeric in the cytosol [32]. Thus Bax is not kept in the cytosol through complex formation with other Bcl-2-family proteins.

Non-ionic detergents have been shown to induce homo- and hetero-complex formation of the Bcl-2-family proteins [32,33]. We show that exposing recombinant Bax to specific detergents induces oligomerization and renders the protein channel-forming activity. Similarly, monomeric Bax in the cytosol of mouse liver shifted into a complex when exposed to OG, although it is not shown that this oligomer is composed of Bax exclusively.

Exposure to detergent induces formation of a Bax oligomer composed of six to eight Bax molecules. The Bax oligomerization appears to be an irreversible process. When the detergent was removed no dissociation into monomers was detected; rather, the Bax oligomers formed larger aggregates. When detergent was added back to the samples the initial oligomers reappeared, showing that formation of the larger aggregates was a reversible process. No association into larger aggregates was detected in monomeric Bax. The altered properties of oligomeric Bax compared with monomeric Bax suggest that detergent exposure induces conformational changes in the protein. Conformational changes in Bax have been detected after detergent treatment and after Bid-induced activation [32,34]. Detergent-sensitive aggregation of oligomeric Bax would be consistent with a model where hydrophobic domains have been demasked and exposed. Such conformational changes could promote targeting and insertion of the protein into lipid membranes. This is supported by the Western-blot analysis of mitochondria treated with Bax. Oligomeric Bax associates more efficiently with the mitochondria compared with monomeric Bax. Interestingly, the Bax protein used in these experiments did not contain the hydrophobic C-terminal domain, suggesting that the conformational changes involve other or additional domains.

Bax homodimer formation has been suggested to be involved in activation of the protein [32,33,35]. However, SDS/PAGE has been used to analyse quaternary structures. Although in this study Bax was present in solution as an oligomer with a molecular mass of 160000 Da, only monomers and dimers were detected on SDS/PAGE (results not shown). In a study by Gross et al. [35] where Bax was cross-linked in the mitochondrial membrane after induction of apoptosis, not only dimers but also higher-molecular-mass complexes, which could correspond to Bax oligomers, were detected.

The behaviour of Bax is reminiscent of the bacterial toxin aerolysin. Aerolysin is present as a monomer or dimer in solution. However, the protein can oligomerize and oligomerization is a prerequisite for insertion into the membrane and for channel formation [36]. A mutant that was unable to form oligomers was inactive in terms of channel formation [36]. The aerolysin oligomer has been shown to be a heptamer, which is also the active unit in the membrane [37]. The molecular mass of the Bax oligomer corresponds to a complex of six to eight molecules. We have shown previously that active C-terminally truncated Bax isolated in the presence of detergent from a glutathione S-transferase fusion protein is present as an oligomer in solution [38]. Equilibrium centrifugation analysis showed the complex to best fit a heptamer model [38]. The large conductance levels, up to 1.6 nS, recorded for the Bax channel in lipid bilayers would be consistent with a large channel [20].

Combined, these results show that recombinant monomeric Bax has no channel-forming activity and does not trigger cytochrome *c* release from mitochondria. Exposure to specific detergents induces oligomerization, which renders the protein channel-forming activity and the ability to trigger mitochondrial cytochrome *c* release. The oligomerization is possibly accompanied by conformational changes that result in exposure of hydrophobic domains, and which could be involved in the targeting and insertion of the protein in lipid membranes. The identity of the triggering factor that results in activation of Bax during apoptosis still remains to be determined.

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