Bax-induced cytochrome *c* release from mitochondria depends on α -helices-5 and -6

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The pro-apoptotic protein Bax plays a key role in the mitochondrial signalling pathway. Upon induction of apoptosis, Bax undergoes a conformational change and translocates to mitochondrial membranes, where it inserts and mediates the release of cytochrome *c* from the intermembrane space into the cytosol. However, the domains of Bax that are essential for the induction of cytochrome *c* release are still elusive. Therefore various Bax deletion mutants were generated and expressed in *Escherichia coli*. The proteins were then purified in order to delineate the function of the transmembrane domain, the BH3 (Bcl-2 homology 3) domain and the putative pore-forming α -helices-5 and -6. These proteins were used to analyse the mechanism of Bax-

induced cytochrome *c* release from mitochondria. None of the Bax proteins caused cytochrome *c* release merely through physical perturbation of the mitochondrial outer membrane. The α -helices-5 and -6 of Bax were shown to mediate the insertion of the protein into mitochondrial membranes and to be essential for the cytochrome *c*-releasing activity of Bax. In contrast, neither the transmembrane domain nor a functional BH3 domain is required for the Bax-mediated release of cytochrome *c* from mitochondria.

Key words: apoptosis, Bax, Bcl-2, cell death, cytochrome *c*, mitochondria.

INTRODUCTION

Apoptosis plays an important role in many physiological processes, including the removal of autoreactive lymphocytes and of redundant cells during development. Apoptosis is also an essential counterbalance to cell division for tissue homeostasis [1]. Having first been defined morphologically, apoptosis is now well defined biochemically. Two major signalling pathways have been delineated, an extrinsic and an intrinsic apoptotic pathway. The two pathways converge on the activation of caspases, a family of intracellular cysteine proteases. Caspases exist as inactive zymogens in the cytosol. Upon induction of apoptosis, these zymogens are proteolytically cleaved to form active heterodimeric enzymes [2]. Active caspases can cleave other caspases, resulting in a proteolytic cascade with the executioner caspases-3, -6 and -7 at the distal end. These executioner caspases cleave cellular substrates, resulting in the typical morphological hallmarks of apoptosis [3].

The extrinsic pathway is triggered by death-domain-containing cell surface receptors of the tumour necrosis factor receptor superfamily, which results in the recruitment and proteolytic activation of caspase-8 as an initiator caspase of a protease cascade [4]. The intrinsic apoptotic signalling pathway involves mitochondria and results in the release of pro-apoptotic factors from the mitochondria, such as cytochrome *c* and Smac [5–7]. Once released into the cytosol, cytochrome *c* induces the formation of the apoptosome, consisting of caspase-9, Apaf-1 (apoptotic protease-activating factor-1) and cytochrome *c*. Subsequently, a caspase cascade is initiated that triggers the proteolytic activation of executioner caspases, such as caspase-3, to perform the final steps in the apoptosis process [5,8–10].

Bcl-2 family proteins are important regulators of apoptosis. They consist of a wide variety of anti-apoptotic proteins such as Bcl-2, but also include pro-apoptotic proteins such as Bax. Bcl-2 was first described in follicular lymphoma, where the t(14;18) chromosomal translocations fuse the *bcl-2* gene to the immunoglobulin enhancer [11]. Subsequently, Bcl-2 has been shown to promote cell survival [12]. Bax, a pro-apoptotic protein member of the Bcl-2 family, participates in the induction of apoptosis in response to many apoptotic signals [13,14]. Overexpression of Bax has been shown to induce apoptosis in a variety of different cellular contexts [13,15].

Amino acid alignments of Bcl-2 family proteins have demonstrated conserved domains, of which BH (Bcl-2 homology) 1, BH2 and BH3 have been characterized as being involved in homo- and hetero-dimerization of these proteins [16]. Bax has been shown to form homodimers and heterodimers with other Bcl-2 family proteins via interaction of the BH1 and BH2 domains of one molecule with the BH3 domain of the other [16–18]. Mutants of Bax involving mutation of the BH3 domain that are incapable of either homodimerizing with themselves or heterodimerizing with other members of the Bcl-2 family have been described. Such mutants are reportedly inactive in promoting cell death in mammalian and yeast cells [18–20].

The three-dimensional structures of the Bcl-2 family members Bcl-X_L, Bid, Bax and Bcl-2 have been resolved, revealing a striking resemblance to the pore-forming domains of certain bacterial toxins [21–23]. This structural similarity correlates with the ability of Bax, Bcl-2, Bcl-X_L and Bid to form ion-conducting pores in synthetic lipid membranes [24–26]. The hairpin pair of α -helices of the bacterial pore-forming domains which share structural similarity with the Bcl-2 family proteins (α -helices-5

Abbreviations used: Bax- Δ C, Bax lacking the C-terminal 20 amino acids; BH, Bcl-2 homology; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; CsA, cyclosporin A; DIABLO, direct IAP (inhibitor of apoptosis protein) binding protein with low pI; PTP, permeability transition pore.

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and -6) participate in membrane insertion, leading to the generation of voltage-dependent ion-conducting channels [27]. The deletion of the corresponding α -helices in Bcl-2 and Bax abrogates their ability to form ion channels, suggesting a similar function [25,26]. The putative pore-forming domains of Bax are amphipathic. Presumably, when inserted into membranes, the polar residues of the amphipathic α -helices line the aqueous channel of the pore, thus mediating the function of the protein [28].

The release of cytochrome *c* from mitochondria upon induction of apoptosis has been shown previously [5]. It has also been shown that the pro-apoptotic protein Bax can trigger the cytochrome *c* release by an as yet unknown mechanism [9,29,30]. Different mechanisms of Bax action upon mitochondria have been proposed, but the roles of the various domains of Bax so far remain relatively unexplored [31,32]. Transfection studies suggest that the BH1, BH2 and BH3 domains are involved in homo- and hetero-dimerization. The BH3 domain of Bax is necessary for apoptosis induction by this protein [15,18]. The putative pore-forming domain consisting of α -helices-5 and -6 has been shown to form ion channels in lipid bilayers [28,33]. We therefore generated Bax proteins lacking the C-terminal transmembrane domain, a functional BH3 domain and/or the putative pore-forming α -helices, in order to evaluate the relevance of these domains to the mechanism of Bax-induced cytochrome *c* release.

EXPERIMENTAL

Antibodies and reagents

All antibodies, i.e. monoclonal anti-Bax (6A7), polyclonal rabbit anti-Bax, monoclonal anti-cytochrome *c*, monoclonal anti-Smac/DIABLO [direct IAP (inhibitor of apoptosis protein) binding protein with low pI] and monoclonal anti-(cytochrome *c* oxidase subunit IV), were purchased from BD Pharmingen (Heidelberg, Germany). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from Bio-Rad (München, Germany). The cytochrome *c* ELISA was purchased from R&D Systems. The ECL[®] Western blotting detection kit was purchased from Amersham Pharmacia Biotech. All other chemicals were purchased from Sigma-Aldrich and were of the highest grade available.

Purification of Bax

Recombinant Bax protein was purified according to Montessuit et al. [34], with modifications. The coding DNA sequence for mouse Bax was cloned with the aid of a 5' terminal *Bgl*II and a 3' terminal *Hind*III restriction site into the prokaryotic expression vector pBAD/HisB (Invitrogen). This cloning strategy introduces an N-terminal His tag for affinity chromatography, an Xpress epitope and an enterokinase cleavage site [35]. The resulting plasmid was checked by sequencing and re-transformed into *Escherichia coli* strain LMG 194 (Invitrogen). Bacteria were cultured in 10 litres of RM medium [1 × M9 salts, 2 % (w/v) casamino acids, 1 mM MgCl₂, 0.4 % (w/v) glucose and 100 µg/ml ampicillin] in a fermenter. The expression of the fusion protein was induced by the addition of L-arabinose (1 g/l) followed by further incubation for 4 h at 30 °C. The bacteria were harvested by centrifugation, yielding 60–70 g of bacteria (wet weight), and stored at –20 °C.

A 35 g portion of bacteria was disrupted in the presence of Triton X-100 by lysozyme addition and sonication. After centrifugation (35 000 g, 30 min, 4 °C), the resulting supernatant was applied for 16 h at 4 °C to a 5 ml Ni²⁺-nitrilotriacetate column

(Amersham Pharmacia Biotech). The protein-loaded column was washed and the protein was eluted in one step by 200 mM imidazole. The Bax-containing fractions were diluted 1:4 (v/v) and applied to an anion-exchange column (6 ml Resource Q; Amersham Pharmacia Biotech). The column was washed and the Bax protein was eluted with a linear gradient from 0 to 500 mM NaCl.

The fractions containing Bax protein were concentrated in the presence of storage buffer P2 [20 mM sodium phosphate (pH 7.5), 5 % (v/v) glycerol and 0.5 % (w/v) octyl glucoside] by ultrafiltration to a final protein concentration of 0.1 mg/ml (VIVA-Spin6 Concentrator, 5 kDa cut off; VIVA Science). The resulting Bax protein was stored in aliquots at –70 °C.

Purification of Bax-ΔC protein

Bax-ΔC (recombinant Bax protein lacking the C-terminal 20 amino acids) was prepared according to Xie et al. [36] with slight modifications. Briefly, *E. coli* XL-1 Blue cells carrying the plasmid pGEX-4T1-Bax-ΔC were grown overnight at 37 °C in 1 litre of TB (Terrific Broth) medium supplemented with 1.5 % (v/v) glycerol containing 0.5 mg/ml carbenicillin. Protein overexpression was induced by the addition of 10 µM isopropyl β-D-thiogalactoside, and cells were cultured overnight at room temperature. The harvested cells were disrupted in the presence of Tween 20 by lysozyme addition and sonification. After centrifugation (27 500 g, 10 min, 4 °C), the supernatant was applied to glutathione-Sepharose (Amersham Pharmacia Biotech) by overnight incubation. After extensive washing, the protein was recovered by proteolytic cleavage with thrombin (Pharmacia). The eluted Bax-ΔC protein was dialysed for 4 h in the presence of 10 mM Tris/HCl (pH 8.0), 1 mM EDTA and 0.1 % (v/v) 2-mercaptoethanol. The dialysed protein was applied to a Resource Q column (Amersham Pharmacia Biotech) and the protein was eluted with a linear gradient from 0 to 500 mM NaCl in dialysis buffer. Fractions containing the Bax-ΔC protein were concentrated in the presence of storage buffer P1 (10 mM Tris/HCl, pH 7.5, 100 mM NaCl) using a stirred Amicon cell (Millipore) to a final protein concentration of 0.3 mg/ml. The Bax-ΔC protein was stored at –70 °C. All proteins were purified under conditions known to induce the conformational change required for oligomerization and the pro-apoptotic effects of the Bax protein [37,38].

Protein analysis and detection

Proteins were separated by SDS/PAGE using standard procedures [39]. Proteins were visualized with a Coomassie Brilliant Blue stain [40].

Edman degradation analysis was performed in the service laboratory of the University of Cologne (ZMMK) after sample preparation, as described in [41]. Western blotting was performed according to standard protocols [42]. SDS/PAGE-separated proteins were transferred on to nitrocellulose membranes (Protran 0.2 µm; Schleicher and Schüll). After blocking, membranes were incubated with the various primary antibodies (VIVA-Spin6 Concentrator, 5 kDa cut off; VIVA Science) followed by horseradish peroxidase-conjugated secondary antibodies and ECL-based detection.

Isolation of mitochondria

Mitochondria were purified from the livers of female rats by differential centrifugation and separation on a sucrose gradient

[43]. Mitochondria were suspended in MSB [400 mM mannitol, 50 mM Tris/HCl (pH 7.2), 10 mM KH_2PO_4 , 0.5 % (w/v) BSA] and kept on ice for up to 4 h.

Cytochrome *c* release

Mitochondria (300 μg) were incubated with recombinant Bax protein (0–1000 nM) in 50 μl of MSB supplemented with 5 mM succinate, 5 μM rotenone and 4 mM MgCl_2 at 30 °C for 30 min. Mitochondria were pelleted by centrifugation at 500 *g* for 10 min and the resulting supernatant was analysed by Western blotting with an anti-cytochrome *c* antibody, an anti-Smac antibody or a cytochrome *c* ELISA (R&D Systems) to determine the amount of cytochrome *c* released [30].

Membrane insertion of Bax proteins

Mitochondria (1200 μg) were incubated with recombinant Bax protein (1700 nM) in 300 μl of MSB supplemented with 5 mM succinate, 5 μM rotenone and 4 mM MgCl_2 at 30 °C for 1 h. Mitochondria were pelleted by centrifugation at 500 *g* for 10 min. The pelleted mitochondria were washed four times with MSB. Loosely attached proteins were removed by incubation of the mitochondria in 0.1 M Na_2CO_3 in MSB on ice for 20 min, as described by Antonsson et al. [44]. After centrifugation for 1 h at 100 000 *g*, the membrane pellet containing inserted proteins was analysed by Western blotting using the polyclonal rabbit anti-Bax antibody [45] or the monoclonal anti-Smac/DIABLO antibody. Use of equal amounts of mitochondria was verified by Western blotting using an antibody that detects cytochrome *c* oxidase (subunit IV).

Haemoglobin release

Erythrocytes were prepared from fresh heparinized human blood by dilution of 0.5 ml of whole blood in 40 ml of PBS (pH 7.4), and centrifugation at 5000 *g* for 10 min at 4 °C. Erythrocytes (100 μg) were incubated with 1000 nM mBax protein or the corresponding buffers in 100 μl of SPB (250 mM sucrose, 20 mM Pipes/NaOH, pH 7.2) at 30 °C for 1 h. Samples were centrifuged at 5000 *g* for 5 min, and the amount of haemoglobin released was analysed by measuring the absorbance at 405 nm using an ELISA Plate-Reader (Dynex Technologies) [24].

Mitochondrial permeability transition

The swelling of mitochondria *in vitro* caused by induction of the PTP (permeability transition pore) was assayed by using mitochondria (1 $\mu\text{g}/\mu\text{l}$) resuspended in CFS (220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 10 mM Hepes/NaOH, pH 7.4, 5 mM KH_2PO_4) supplemented with 5 mM succinate and 5 μM rotenone. Mitochondria were treated with 150 μM CaCl_2 in the presence or absence of 4 mM MgCl_2 , with recombinant Bax protein or the corresponding volume of buffer. The variation in absorbance caused by swelling was measured at 30 °C using a double-beam spectrophotometer (Lambda 40; Perkin Elmer) at 540 nm [46].

Mitochondrial membrane potential

The mitochondrial membrane potential was measured *in vitro* by using the cationic membrane-permeable dye, safranin [47]. Safranin either accumulates in the mitochondrial matrix or aggregates with the surface of the inner membrane, depending on

the membrane potential. Diffusion potentials for potassium and hydrogen ions over the inner membrane from isolated mitochondria induce a modification of the spectral properties of safranin. This variation correlates linearly with the membrane potential. A decrease in potential results in a decrease in absorbance at 555 nm. Mitochondria (0.3 $\mu\text{g}/\mu\text{l}$) were resuspended in KCl medium (150 mM KCl, 10 mM Hepes/NaOH, pH 7.4, 1 mM GSH, 0.1 mM EGTA) supplemented with 10 mM K_2HPO_4 , 5 mM L-glutamate, 4 mM L-malate, 2 mM MgCl_2 and 10 $\mu\text{g}/\text{ml}$ safranin. Mitochondria were treated with recombinant Bax protein or the corresponding volume of buffer. The uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) (50 μM) was used to induce depolarization of the membrane potential. The absorbance variation caused by the Bax protein or CCCP was measured at 30 °C using a double-beam spectrophotometer (Lambda 40; Perkin Elmer) at 555 nm.

Electron microscopy

Mitochondria were resuspended in CFS supplemented with 5 mM succinate and 5 μM rotenone, and treated either with 300 nM Bax protein or with 150 μM CaCl_2 in the presence or absence of 10 μM CsA (cyclosporin A), as described for the mitochondria permeability transition assay [48,49]. After 1 h, 10 μl aliquots of mitochondrial preparations from each experiment were transferred into Microfuge tubes and pelleted by centrifugation. The pelleted mitochondria were fixed immediately in 2.5 % (v/v) glutaraldehyde (Sigma, electron microscopy grade, distilled) prepared in 0.2 M sodium cacodylate buffer (pH 7.4; 359 mOsm). The pellets were washed in 0.2 M sodium cacodylate buffer and processed with epoxy resin (TAAB 812) in a Lynx tissue processor (Leica Microsystems UK Ltd). Ultra-thin sections (60 nm) were cut using a Leica Ultracut S ultra-microtome and stained with uranyl acetate and lead citrate (all reagents from BDH). The sections were examined with a Philips CM10 transmission electron microscope operating at 80 kV. Four random fields were recorded at a magnification of $\times 3900$, and from each of these a random field at was recorded $\times 11500$.

RESULTS

Purification of Bax proteins

In order to perform a structure–function analysis of Bax protein with regard to its ability to release cytochrome *c* from isolated mitochondria, Bax and Bax deletion mutants were cloned into expression vectors and expressed in *E. coli* (Figure 1A). Bax consists of nine α -helices, with α -helices-1–8 resembling Bcl- X_L . The C-terminal α -helix-9 occupies the hydrophobic pocket suggested previously to mediate the formation of homo- and hetero-dimers via the BH3 domain [35]. Proteins with deletions in the transmembrane domain (α -helix-9) alone (Bax ΔC), the transmembrane domain plus the BH3 domain (α -helix-2) (Bax $\Delta\text{IGDE};\Delta\text{C}$) or the transmembrane domain plus pore-forming α -helices-5 and -6 (Bax $\Delta\text{h5/6};\Delta\text{C}$), or with a combination of all deletions (Bax $\Delta\text{IGDE};\Delta\text{h5/6};\Delta\text{C}$), were purified according to the protocol established for Bax ΔC . Figure 1(B) depicts purified Bax protein and the Bax ΔC deletion mutants after Coomassie Blue staining and Western blotting. All Coomassie Blue-stained protein bands were also detected by the rabbit anti-Bax antibody [45]. A monoclonal antibody specific for the N-terminal amino acids 12–24 of Bax (6A7) stained only the main product band, full-length Bax ΔC (indicated by ** in Figure 1B), indicating that the smaller fragments are degradation products caused by N-terminal cleavage (results not shown). This was also

Figure 1 Purification of Bax proteins

(A) Schematic overview of recombinant Bax proteins. TM, transmembrane domain. (B) After separation, recombinant Bax proteins lacking the transmembrane domain were stained by Coomassie Blue and detected by Western blotting using a polyclonal primary antibody against Bax (rabbit anti-Bax) followed by a horseradish peroxidase-conjugated secondary antibody and ECL. Molecular masses (MW) are indicated on the right.

confirmed by N-terminal protein sequencing, which identified amino acid 22 of Bax as the N-terminus in the smallest fragment (indicated by * in Figure 1B), whereas the N-terminal amino acids of full-length BaxΔC were confirmed to be present in the most prominent protein band (results not shown). Bax has been reported to be cleaved N-terminally *in vivo*, which enhances its pro-apoptotic effect [50]. The presence of small amounts of N-terminal degradation products is not expected, therefore, to decrease the cytochrome *c*-releasing activity of the Bax protein.

The transmembrane domain of Bax is not required for induction of cytochrome *c* release

These mutant Bax proteins enabled us to study the roles of various domains in the release of cytochrome *c* from mitochondria. Wild-type Bax protein and BaxΔC were first compared with regard to their ability to release cytochrome *c* from mitochondria (Figure 2A). Both Bax and BaxΔC induced the release of cytochrome *c* from mitochondria in a concentration-dependent manner (Figure 2B). The dose–response relationship of BaxΔC seems indistinguishable from that of Bax. In addition, the detergent Triton X-100 was used for releasing the total amount of cytochrome *c* from an equal volume of mitochondria. Compared with this positive control, Bax at every concentration used released a lower amount of cytochrome *c*, indicating that Bax does not merely solubilize the mitochondria like the detergent, but induces a more specific release of cytochrome *c*. The results obtained by Western blotting were quantified by an ELISA specific for cytochrome *c*, using the supernatants of Bax-treated mitochondria. As shown in Figure 2(C), Bax and BaxΔC released cytochrome *c* from the mitochondria in a similar fashion. This indicates that the transmembrane domain of Bax is not required for its cytochrome *c*-releasing activity.

Figure 2 Release of cytochrome *c* from isolated mitochondria by Bax

(A) Isolated rat liver mitochondria were treated for 30 min at 30 °C with 200 nM Bax or BaxΔC. As a negative control, mitochondria were left untreated or treated with storage buffer (P2). After centrifugation, supernatants were removed and analysed by Western blotting with a monoclonal antibody against cytochrome *c*. An aliquot of mitochondria was lysed by the detergent Triton X-100 to determine the total content of cytochrome *c*. (B) Mitochondria were incubated as in (A) with increasing concentrations of Bax or BaxΔC. As a negative control (0 nM), the mitochondria were treated with storage buffer (P2). The resulting supernatants were analysed by Western blotting. (C) The supernatants from (A) were also analysed by ELISA ($n=2$). The corresponding amount of cytochrome *c* released by lysis with Triton X-100 was 11 020 ng/ml.

Role of a functional BH3 domain and of α -helices-5 and -6 in Bax-mediated cytochrome *c* release

As the C-terminal transmembrane domain of Bax (α -helix-9) is not responsible for the Bax-induced release of cytochrome *c* from isolated mitochondria, we next investigated whether a functional BH3 domain (α -helix-2) or the putative pore-forming domain (α -helices-5 and -6) of Bax are required for the induction of cytochrome *c* release from mitochondria. The Bax-dependent cytochrome *c* release came to an end within 10 min. However, we incubated the proteins with the mitochondria for 30 min, to allow for possible slower membrane incorporation of the mutated proteins. BaxΔC mutants lacking the amino acid motif IGDE in the core region of the BH3 domain (BaxΔIGDE;ΔC), the putative pore-forming domain (BaxΔh5/6;ΔC), or both domains (BaxΔIGDE;Δh5/6;ΔC) were tested in parallel. All three proteins were expressed and purified without the C-terminal transmembrane domain, because the deletion of this final α -helix resulted in larger yields of concentrated proteins, which were soluble in buffers without a requirement for detergent. As shown in Figure 3(A), Bax lacking a functional BH3 domain (BaxΔIGDE;ΔC) and BaxΔC released similar amounts of cytochrome *c*. In contrast, Bax mutants lacking α -helices-5 and -6 (BaxΔh5/6;ΔC) or lacking both domains (BaxΔIGDE;Δh5/6;ΔC) failed to release significant amounts of cytochrome *c*.

To analyse the activity of Bax mutants lacking α -helices-5 and -6, we performed dose–response experiments measuring the release of cytochrome *c* from isolated mitochondria. The mutant BaxΔIGDE;Δh5/6;ΔC protein completely failed to release

Figure 3 The putative pore-forming domain of Bax is important for cytochrome *c* release

(A) Isolated rat liver mitochondria were treated for 30 min at 30 °C in the presence of 0.5 μ M of the various Bax mutants (see Figure 1A). As a negative control, mitochondria were treated with storage buffer (P1). After centrifugation, supernatants were removed and analysed by Western blotting with a monoclonal antibody against cytochrome *c*. (B) Isolated rat liver mitochondria were incubated with increasing concentrations of mutant Bax proteins lacking α -helices-5 and -6. As a negative control, mitochondria were treated with storage buffer (P1). The resulting supernatants were analysed by Western blotting. (C) Mitochondria were incubated with increasing concentrations of the various Bax proteins lacking the C-terminal transmembrane domain. As a negative control (0 nM), mitochondria were treated with storage buffer (P1). Triton X-100-lysed mitochondria were used to determine the total cytochrome *c* content. The resulting supernatants were analysed by ELISA ($n = 3$). The total cytochrome *c* content was 12 050 ng/ml.

cytochrome *c* at all doses tested, whereas Bax Δ h5/6; Δ C induced the release of minor amounts of cytochrome *c* at higher concentrations (Figure 3B). Supernatants were also analysed by ELISA (Figure 3C). As expected, the Bax mutants lacking the putative pore-forming domain of Bax displayed dramatically reduced cytochrome *c*-releasing ability compared with Bax Δ IGDE; Δ C. Notably, as quantified by ELISA, all Bax proteins released significantly lower amounts of cytochrome *c* compared with the Triton X-100 detergent control, indicating that the Bax-induced cytochrome *c* release is not secondary to non-specific effects such as outer membrane rupture. These data indicate that a functional BH3 domain is of minor importance for Bax-induced cytochrome

Figure 4 α -Helices-5 and -6 mediate insertion into the mitochondrial membrane

(A) Isolated rat liver mitochondria were incubated for 60 min at 30 °C in the presence of the various Bax mutant proteins lacking the C-terminal transmembrane domain (see Figure 1A). As a negative control, the mitochondria were left untreated. The mitochondria were then pelleted and washed. Attached proteins were removed by treatment with 0.1 M Na_2CO_3 . After ultracentrifugation at 100 000 g the membrane pellet with the inserted proteins was resuspended in SDS/PAGE loading buffer. The presence of inserted Bax proteins was analysed by Western blotting using a polyclonal antibody against Bax (rabbit anti-Bax). Released and mitochondrial Smac/DIABLO protein was detected by Western blotting using a monoclonal antibody. As a control, the blot was probed with an antibody for the integral mitochondrial membrane protein cytochrome *c* oxidase (subunit IV). (B) Human erythrocytes were isolated and incubated with 1 μ M Bax protein at 30 °C for 1 h. The assay buffer and the storage buffers (P1 or P2) were used as negative controls. After centrifugation, the amount of haemoglobin released was analysed by spectrophotometry at 405 nm. For the estimation of the total amount of haemoglobin, erythrocytes were lysed with 1% (v/v) Triton X-100 (= 100%). The amount of haemoglobin released is expressed as a percentage of the total amount present ($n = 2$).

c release. In contrast, the presence of the putative pore-forming domain of Bax, i.e. α -helices-5 and -6, is of great importance for cytochrome *c*-releasing activity.

Interaction of Bax with membranes

To investigate how Bax proteins interact with membranes, we performed assays of insertion into mitochondrial membranes (Figure 4A). In order to detect the inserted Bax protein with the highest possible sensitivity, a concentrated suspension of mitochondria was incubated with highly concentrated Bax protein to facilitate as much insertion of Bax as possible. In untreated

Figure 5 Influence of Bax proteins on the mitochondrial permeability transition

(A) The morphology of untreated isolated mitochondria and mitochondria treated with Bax proteins or CaCl₂ in the presence or absence of CsA was assessed by electron microscopy. Mitochondria treated with 300 nM full-length Bax or BaxΔC did not show altered morphology when compared with untreated samples. Swelling induced by CaCl₂ (150 μM) was fully reversed by the addition of 10 μM CsA. Original magnification × 15 000. (B) Isolated rat liver mitochondria were resuspended in CFS medium. The absorbance at 30 °C was measured in a double-beam spectrophotometer at 540 nm over time. A permeability transition was induced by the addition of 150 μM CaCl₂ (black), and was inhibited in the presence of 4 mM MgCl₂ in the buffer (grey), upper left panel. At the indicated times, proteins were added to a final concentration of 50 nM Bax, 500 nM BaxΔC or 340 nM BaxΔIGDE;ΔC (black). The corresponding volume of storage buffer (P2 or P1) (grey) was added to controls.

isolated rat liver mitochondria, no endogenous Bax could be detected. Under conditions where BaxΔC induced cytochrome *c* release from isolated mitochondria, BaxΔC was found to be inserted into the mitochondrial membranes. Bax lacking a functional BH3 domain (BaxΔIGDE;ΔC) was also inserted into membranes. In contrast, neither BaxΔh5/6;ΔC nor BaxΔIGDE;Δh5/6;ΔC was inserted into mitochondrial membranes. In addition, we were able to show that the insertion of the proteins BaxΔIGDE;ΔC and BaxΔC was accompanied by the release of mitochondrial Smac/DIABLO into the supernatant of Bax-treated isolated mitochondria.

To rule out a non-physiological lysis of membranes by Bax, the ability of the Bax proteins to lyse erythrocyte membranes was investigated. When plasma membranes of erythrocytes are lysed with detergent, haemoglobin is released, and this release can be assessed by spectrophotometry. As shown in Figure 4(B), none of the Bax proteins induced any release of haemoglobin, indicating that the Bax mutants do not lyse membranes in a non-specific manner.

Bax does not induce either swelling of mitochondria or changes in the mitochondrial membrane potential

The mechanism by which Bax induces the release of cytochrome *c* from mitochondria is still controversial [32]. In some circumstances the opening of the PTP, with the subsequent osmolarity changes leading to swelling of the mitochondria and disruption of the outer membrane, have been implicated in cytochrome *c* release [51]. To investigate morphological changes induced by Bax, mitochondria treated with Bax or BaxΔC were assessed by electron microscopy (Figure 5A). CaCl₂, a known inducer of mitochondrial PTP formation, was used in control experiments, and induced swelling with complete destruction of internal cristae. This process was blocked by the PTP inhibitor CsA. We observed that the mitochondrial PTP-induced swelling was a fast process, reaching its maximum level within 6 min. In contrast with this, neither Bax nor BaxΔC induced any visible morphological changes in mitochondria, indicating that PTP formation is an unlikely

Figure 6 Influence of Bax proteins on the mitochondrial membrane potential

Isolated rat liver mitochondria were resuspended in KCl medium containing 10 μ g/ml safranin. The absorbance at 30 °C was measured in a double-beam spectrophotometer at 555 nm over time. As a positive control, the decoupler CCCP was added to a final concentration of 50 μ M after 6 min. The decrease in the mitochondrial membrane potential results in an absorbance decrease. At the indicated times, protein was added to a final concentration of 500 nM (black). The corresponding volume of storage buffer (P1) (grey) was added to the controls.

explanation for the mechanism of Bax-induced cytochrome *c* release.

In parallel, mitochondrial swelling was measured using a double-beam spectrophotometer. Concurrent with the results shown in Figure 5(A), CaCl_2 treatment induced mitochondrial swelling, which was inhibited in the presence of MgCl_2 . In contrast, none of the Bax proteins that were able to release cytochrome *c* induced mitochondrial swelling, indicating that opening of the PTP was unlikely to have caused the cytochrome *c* release.

In addition, the influence of Bax on the mitochondrial membrane potential was measured (Figure 6). Mitochondria were loaded with the cationic membrane-permeable dye safranin, which can be used to monitor changes in the membrane potential in isolated mitochondria [47]. Loss of membrane potential can be followed spectroscopically, resulting in a decrease absorbance. When mitochondria were incubated with Bax mutants, the absorbance remained stable and hence the membrane potential was not disturbed. This contrasts with the membrane potential loss induced by the proton ionophore CCCP. The conclusion suggested by these data is that neither Bax Δ C nor the Bax mutants lacking various domains induce a permeability transition in isolated mitochondria.

DISCUSSION

To delineate the domains of Bax involved in the release of cytochrome *c* from mitochondria, we expressed Bax proteins lacking the transmembrane domain, a functional BH3 domain and/or the putative pore-forming domain, and investigated their function in cytochrome *c* release from isolated mitochondria. Here we show that Bax lacking the transmembrane domain can induce the release of cytochrome *c* to the same extent as full-length Bax. Furthermore, Bax lacking a functional BH3 domain also induced

the release of cytochrome *c*, indicating that the BH3 domain is of minor importance for cytochrome *c* release from mitochondria. In contrast, deletion of the putative pore-forming domain of Bax, comprising α -helices-5 and -6, prevented insertion of Bax into mitochondrial membranes and greatly decreased the cytochrome *c*-releasing activity of Bax, indicating that this domain is crucial for Bax-induced cytochrome *c* release from mitochondria. Concomitantly, deletion of the putative pore-forming domain of Bax also prevented the release of Smac/DIABLO from mitochondria. The results of our present study show that Bax induces the release of cytochrome *c* in a specific, controlled and dose-dependent manner, without any evidence of mitochondrial swelling.

It has been shown previously that proteins of the Bcl-2 family can be targeted to membranes via a hydrophobic transmembrane domain at their C-terminus. For example, the putative transmembrane domain of Bcl-2 functions as a signal anchor that targets the protein to the mitochondrial outer membrane, the endoplasmic reticulum and the nuclear envelope [52–55]. Deletion of this transmembrane region resulted in attenuation of the binding and function of Bcl-2 [54,56,57]. However, the nature of the targeting signal used for the restricted subcellular localization of Bcl-2 has yet to be determined, and little is known in this regard about other members of the Bcl-2 family.

A common mechanism for the membrane insertion of Bcl-2 and Bax has been assumed, based on structural similarities and co-localization [58]. The pro-apoptotic Bax protein also contains a predicted transmembrane domain at its C-terminus. The ability of Bax to translocate to mitochondrial membranes is regulated, and depends on a death signal received by the cell [59,60]. In unstimulated cells, Bax is typically located in the cytosol [59,60]. The N-terminal domain of Bax prevents its insertion into mitochondrial membranes in non-apoptotic cells. The regulated targeting of Bax to mitochondria and its insertion into the mitochondrial outer membrane appear to be mediated by a

defined C-terminal polypeptide [61]. In contrast, other groups have shown that deletion of the C-terminus of Bax did not abolish mitochondrial targeting, and therefore proposed that the C-terminus is not a mitochondrial targeting signal [62]. Transfection experiments also suggest that deletion of the membrane-anchoring domain of Bax does not impair its targeting to mitochondria or the induction of apoptosis, which probably involves dimerization with endogenous Bax protein [15]. Furthermore, Cartron et al. [63] recently suggested that the N-terminus of Bax is responsible for mitochondrial targeting. Therefore the importance of the C-terminal transmembrane domain of Bax (α -helix-9) remains controversial. In contrast with other studies, our experiments employing isolated mitochondria enabled the examination of Bax insertion into mitochondrial membranes and Bax-induced release of cytochrome *c* independent of upstream processes such as Bax targeting. Thus, while the transmembrane domain of Bax might be indispensable for the targeting of Bax to mitochondria, our data clearly show that this domain is irrelevant for the Bax-mediated induction of cytochrome *c* release from mitochondria.

The cytochrome *c*-releasing activities of selected Bax deletion mutants also revealed that the amino acids Ile-Gly-Asp-Glu (IGDE) within the core region of the BH3 domain of Bax are of minor importance for the release of cytochrome *c*. It was reported previously that deletion of these four well conserved amino acids from Bax ablated its ability to homodimerize and to induce apoptosis when transfected into HEK-293 or Rat-1 cells [15]. That study also suggested that both BH3-mediated dimerization and targeting to mitochondrial membranes were essential for the apoptotic function of Bax in yeast and mammalian cells [15]. We conclude that, despite the role of the BH3 domain in Bax oligomerization or dimerization in response to apoptotic signals, it is not required for insertion into mitochondrial membranes or for cytochrome *c* release. Thus, while BH3-mediated dimerization or oligomerization may be involved in Bax activation, it is not essential for the cytochrome *c* release mechanism.

The presence of a pore-forming domain of Bax, Bcl-2, Bcl-X_L and Bid correlates with the ability of these proteins to form ion-conducting pores in synthetic lipid membranes [24–26]. By analogy with bacterial colicins, it is speculated that two structurally related α -helices in the Bcl-2-family proteins (α -helices-5 and -6) form a hairpin structure that participates in membrane insertion. The deletion of α -helices-5 and -6 from Bax abrogates its ability to form ion channels *in vitro*, suggesting a similar function as the ion-conducting channels created by bacterial colicins [25–27,64]. We have shown here that the same domain, i.e. α -helices-5 and -6, permits the insertion of Bax that is required for subsequent release of cytochrome *c* into mitochondrial membranes. Presumably, when inserted into membranes, the polar residues of the amphipathic α -helices line the aqueous channel of the pore, thus mediating the function of the protein [28]. Alternatively, this pore-forming domain might interact with other mitochondrial proteins to form the cytochrome *c* channel.

In view of their similar structure and proposed function, bacterial colicins and Bax might be derived from a common evolutionary ancestor. In this regard, it was hypothesized recently that the mechanisms of programmed cell death might have their origin in α -proteobacterial endosymbionts, which used proteases to kill their host cells [65,66].

Taken together, the present observations, in combination with previously published results, suggest separate functions for the well defined domains of Bax protein in the Bax-mediated apoptosis pathway. (1) The putative pore-forming domain comprising α -helices-5 and -6 is important for the cytochrome *c*-releasing activity of Bax. These α -helices insert into mito-

chondrial membranes and presumably form a pore, either by themselves or in co-operation with other proteins, which mediates cytochrome *c* release. (2) The BH3 domain is not directly involved in cytochrome *c* release, but presumably contributes to the apoptotic function of Bax by interacting with regulatory proteins upstream of the cytochrome *c*-releasing step in the apoptotic cascade. The BH3 domain may be required for the homodimerization of Bax at the mitochondria and/or for the binding of other proteins, such as Bid, that are known to activate the Bax protein [67–69]. (3) The transmembrane domain of Bax is dispensable for the induction of cytochrome *c* release by this protein. In addition, Bax can insert into mitochondrial membranes in the absence of the transmembrane domain, via the pore-forming domain. Thus the transmembrane domain might only support the first contact of Bax with mitochondrial membranes, which might then be stabilized and maintained by α -helices-5 and -6.

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