

# Oligomeric C-terminal truncated Bax preferentially releases cytochrome *c* but not adenylate kinase from mitochondria, outer membrane vesicles and proteoliposomes

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**Abstract** The mechanism by which the proapoptotic protein Bax releases cytochrome *c* from mitochondria is not fully understood. The present work approaches this problem using C-terminal truncated oligomeric Bax (BaxΔC). Micromolar concentrations of BaxΔC released cytochrome *c* from isolated rat heart and liver mitochondria, while the release of adenylate kinase was not significantly affected. BaxΔC also released cytochrome *c* but not adenylate kinase from outer membrane vesicles filled with these proteins. However, BaxΔC was ineffective in releasing cytochrome *c* when outer membrane vesicles were obtained in the presence of glycerol, conditions under which the number of contact sites was drastically reduced. BaxΔC did not liberate encapsulated cytochrome *c* and adenylate kinase from pure phospholipid vesicles or vesicles reconstituted with porin. However, when the hexokinase–porin–adenine nucleotide translocase complex from brain mitochondria was reconstituted in vesicles, BaxΔC released internal cytochrome *c* but not adenylate kinase. In all these systems, only a small portion of total cytochrome *c* present in either mitochondria or vesicles could be liberated by BaxΔC. BaxΔC also increased the accessibility of external cytochrome *c* to either oxidation by complex IV or reduction by complex III in intact liver and heart mitochondria. **Conclusions:** (1) BaxΔC selectively releases cytochrome *c* and enables a bidirectional movement of cytochrome *c* across the outer mitochondrial membrane. (2) A multiprotein complex that resembles the mitochondrial contact sites is a prerequisite for BaxΔC action. (3) A limited pool of cytochrome *c* becomes the first target for BaxΔC. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Cytochrome *c*; Adenylate kinase; Bax; Bcl-2; Contact site; Outer membrane; Mitochondrion; Apoptosis

## 1. Introduction

One of the important steps in the process of programmed cell death, or apoptosis, is the release of several proteins, including apoptosis-inducing factor (AIF) [1,2] and cytochrome *c* [3–5], from the mitochondrial intermembrane space into the cytoplasm. There are several stimuli which cause cytochrome *c* release. One of them is the proapoptotic Bcl-2 family member Bax (for review see [6]), which is synthesized in the cytoplasm and translocated to mitochondria [7] where it releases cytochrome *c* [8,9].

The mechanism by which Bax releases cytochrome *c* from the intermembrane compartment to the cytoplasm is still controversial. In earlier investigations, it has been assumed that Bax induces mitochondrial permeability transition, leading to swelling of mitochondria, rupture of the outer membrane and release of several factors into the cytoplasm [10–13]. However, recently most researchers agree that the release of cytochrome *c* from mitochondria during the initial stage of apoptosis, mediated by Bax, is a unique process, not related to disruption of the outer mitochondrial membrane (for reviews see e.g. [14,15]). The mechanism of this process, its extent, and kinetics remain largely controversial. Some authors [12,16,17] observed a limited release of mitochondrial cytochrome *c*, whereas others [18–20] found a massive and rapid release. It has been found that some cells can recover after cytochrome *c* release [19,21] and that the mitochondrial transmembrane potential remains high [8,19,20]. The latter observation strongly argues for the preservation within the mitochondria of a part of cytochrome *c*, sufficiently high for undisturbed functioning of the respiratory chain.

The point of controversy remains whether Bax releases cytochrome *c* in a specific way or whether it can also enable the passage of other proteins across the membrane; in other words, whether Bax may form non-selective pores large enough for protein trafficking through membranes or its effect is limited to cytochrome *c* and, possibly, to other mitochondrial proteins participating in apoptosis, like AIF. The ability of Bax to form pores permeable to small molecules has been documented for isolated mitochondria independently of the permeability transition pore [8,9] and for phospholipid bilayers [22–26]. Some authors observed that some other proteins located in the mitochondrial intermembrane compartment, like adenylate kinase [13,18,27–30], sulfite oxidase

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**Abbreviations:** ANT, adenine nucleotide translocase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

[18], pro-caspase-3 [29] and chaperone proteins [29], are released along with cytochrome *c* from mitochondria within intact cells under conditions that lead to apoptosis or from isolated mitochondria as effect of Bax action. Another analogous proapoptotic protein Bid has been shown to liberate trypsin encapsulated in liposomes [31]. On the other hand, Doran and Halestrap [16] claim that adenylate kinase is not released from Bax-containing rat liver mitochondria under conditions when cytochrome *c* is released. One of the aims of the present investigation was therefore to re-examine the problem of the specificity, or its lack, of the release of cytochrome *c* by the proapoptotic protein Bax.

Another question was whether the release of cytochrome *c* from mitochondria is a unidirectional process or whether external cytochrome *c* can also enter the mitochondrial intermembrane compartment. The recovery of some types of cells after loss of mitochondrial cytochrome *c* [19,21] indirectly points to the latter possibility. On the other hand, however, Kluck et al. [18] observed only a limited accessibility of external cytochrome *c* to complexes III and IV of the respiratory chain of *Xenopus* egg mitochondria in the presence of Bax unless a cytoplasmic proteinaceous factor, termed 'permeability enhancing factor', was also present. Doran and Halestrap [16] described both oxidation of external ferrocytochrome *c* and reduction of external ferricytochrome *c* by rat liver mitochondria containing endogenous Bax and have proposed that permeabilization of the outer membrane to cytochrome *c* by Bax is bidirectional. The present results confirm that notion using purified Bax protein.

Finally, we wanted to approach the problem of whether the permeabilization of the outer mitochondrial membrane differentiates between reduced and oxidized forms of cytochrome *c*.

Methods of expressing Bax and Bcl-2 in bacteria [22,26] made available substantial amounts of these proteins and enabled to simulate apoptotic conditions in experiments with isolated mitochondria. Full length Bax is a hydrophobic protein that requires the presence of detergents in order to be kept in solution, a condition which presents problems when studying permeabilization of biological membranes. Therefore, in the present study we used recombinant truncated Bax, missing the hydrophobic C-terminal part (Bax $\Delta$ C), that is soluble in aqueous media without detergent. This modified proapoptotic protein has been previously shown [24] to be qualitatively as effective in releasing cytochrome *c* as full length Bax.

## 2. Materials and methods

### 2.1. Materials

Oligomeric C-terminal truncated Bax (Bax $\Delta$ C) was prepared as described previously [26]. The concentration of the oligomer will be expressed throughout this paper on the basis of its monomeric molecular mass of 22 kDa [26]. Bcl-2 was prepared according to the procedure outlined previously [22]. Porin was isolated from rat liver mitochondria as described by de Pinto et al. [32]. Cytochrome *c* from horse heart and adenylate kinase from rabbit muscle were from Sigma (St. Louis, MO, USA).

### 2.2. Isolation of liver and heart mitochondria

Male Wistar rats 3–4 months old were used throughout. The livers were homogenized in buffered sucrose–mannitol medium containing EGTA and bovine serum albumin and subjected to differential centrifugation as described previously [33]. Heart mitochondria were isolated essentially as described by Schaller et al. [34] without the trypsin digestion procedure. In brief, pieces of minced hearts were gently ho-

mogenized in 180 mM KCl+10 mM EDTA (pH 7.4) at 0°C, the debris was removed by centrifugation at 600 $\times$ g for 3 min, and mitochondria were sedimented and washed (in 180 mM KCl without EDTA) at 2500 $\times$ g for 10 min. Only mitochondrial preparations with mostly intact outer membranes, assessed by accessibility of the inner membrane to external cytochrome *c* [35] not exceeding 10% for liver and 20% for heart, were used.

### 2.3. Isolation of the outer mitochondrial membrane and preparation of outer membrane vesicles

The outer membrane of rat liver mitochondria was isolated according to Sottocasa et al. [36] with a subsequent modification [37] by disrupting the mitochondria by osmotic shock, hypertonic shrinkage and mild sonication followed by a discontinuous sucrose density gradient centrifugation. To obtain outer membrane vesicles with entrapped cytochrome *c*, 1.5 mM cytochrome *c* was added to the shrinkage–sonication medium. To fill the vesicles with adenylate kinase, the vesicles collected from the density gradient centrifugation were again briefly sonicated in a small volume of the sonication medium containing about 125 U rabbit muscle adenylate kinase per ml and separated from the external medium by exclusion chromatography on Sephadex G-75. Some preparations of the outer membrane vesicles were obtained in the presence of 30% glycerol in the shrinkage medium in order to dissociate contact sites between the two mitochondrial membranes [38].

### 2.4. Isolation of the hexokinase–porin–adenine nucleotide translocase (ANT) complex and its reconstitution into liposomes

The hexokinase–porin–ANT complex, displaying properties of the mitochondrial permeability transition pore [39,40] and considered to be the core of contact sites between the outer and inner mitochondrial membranes, was isolated from rat brains as described previously [39,40]. It was then reconstituted into asolectin vesicles containing 2% cholesterol also as described [39,40]. In brief, the hexokinase-containing fractions were mixed with the liposomes, the remnants of Triton X-100 were removed by addition of Bio-beads SM-2 (Bio-Rad) and the mixture was dialyzed overnight against 180 mM KCl+20 mM HEPES (pH 7.4). Isolated porin was reconstituted in the same way. To fill the proteoliposomes thus obtained with cytochrome *c* or adenylate kinase, they were suspended in a small volume of the medium containing either cytochrome *c* or adenylate kinase, briefly sonicated and separated from the external proteins by chromatography on Sephadex G-75, as described for outer membrane vesicles.

### 2.5. Assays for the release of cytochrome *c* and adenylate kinase

Cytochrome *c* was measured spectrophotometrically either at 408 nm ( $\gamma$  or Soret band) [24] or, after reduction with ascorbate, at 550 nm ( $\alpha$  band) in the supernatants after removal of mitochondria by centrifugation (2500 $\times$ g for 10 min in 1 ml Eppendorf tubes) and filtration through cellulose acetate membranes (0.2  $\mu$ m, Iwaki, Japan) or after removal of outer membrane vesicles or of reconstituted proteoliposomes by centrifugation (400 000 $\times$ g for 45 min in 0.7 ml tubes of a Sorvall RC M100 centrifuge). Adenylate kinase was measured in the same supernatants in the direction 2 ADP $\rightarrow$ ATP+AMP in a coupled optical enzyme assay with glucose+hexokinase+NAD+glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*; Roche, Mannheim, Germany). The determination of succinate dehydrogenase activity was performed as described by King [41].

### 2.6. Oxidation by mitochondria of external added cytochrome *c*

Oxygen uptake was measured using a Clark-type oxygen electrode (YSI, Yellow Springs, OH, USA) equipped with a home-made electronic device to plot the first derivative of the O<sub>2</sub> concentration trace (equivalent to the O<sub>2</sub> uptake rate). Externally added cytochrome *c* was maintained in the reduced state by ascorbate.

### 2.7. Reduction of external added cytochrome *c*

This was followed spectrophotometrically in a dual wavelength mode at 550 versus 540 nm (Shimadzu UV-3000 spectrophotometer).

### 2.8. Statistics

Unless stated otherwise, mean values for at least three experiments  $\pm$  S.D. are shown in the figures. Statistical significance was calculated from the Student's *t*-test.

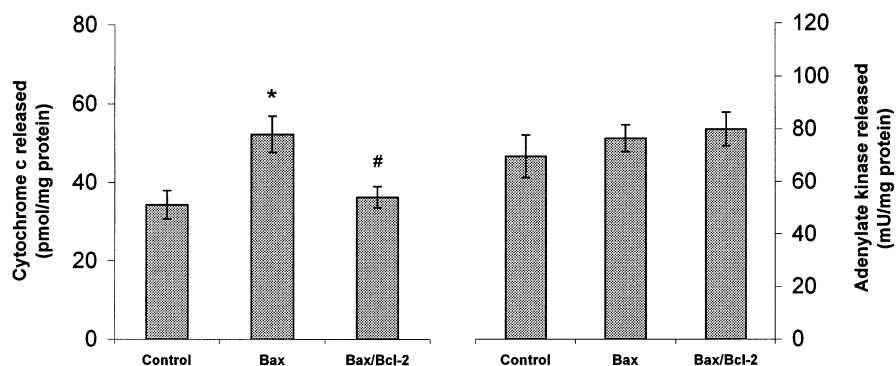


Fig. 1. Effect of Bax $\Delta$ C on the release of cytochrome *c* and adenylate kinase from rat heart mitochondria. Bax $\Delta$ C and Bcl-2 were used at 7.2  $\mu$ M each. Other conditions were as described under Section 2. The total amount of cytochrome *c* in these mitochondrial preparations was  $232 \pm 6$  nmol/mg protein and that of adenylate kinase  $447 \pm 59$  mU/mg protein. \*Statistically significant difference ( $P < 0.02$ ) with respect to the control; #statistically significant difference ( $P < 0.02$ ) with respect to Bax $\Delta$ C.

### 3. Results

#### 3.1. Bax $\Delta$ C facilitates the release of cytochrome *c* but not of adenylate kinase from intact heart mitochondria and outer membrane vesicles

When freshly isolated rat heart mitochondria were incubated at 25°C in a slightly hyperosmotic saline medium, a small but detectable amount of cytochrome *c*, amounting to 0.01% of total cytochrome *c*, was liberated to the medium during 30 min, apparently due to a partial damage to the outer membrane. The release of adenylate kinase was higher and amounted to about 16% of the total content of this enzyme in mitochondria (that could be liberated after hypotonic swelling or solubilization of mitochondria with digitonin). This relatively high amount of adenylate kinase released could be due to the fact that isolated heart mitochondria contained some adsorbed cytosolic enzyme. Bax $\Delta$ C increased the release of cytochrome *c* but not that of adenylate kinase (Fig. 1). Bcl-2, added in a 1:1 ratio, significantly suppressed the Bax $\Delta$ C-dependent cytochrome *c* liberation.

Selective, though partial, release by Bax $\Delta$ C of cytochrome *c* but not of adenylate kinase was also observed in outer membrane vesicles. In this experiment the outer membranes isolated from rat liver mitochondria by the swelling–shrinkage–sonication procedure form closed vesicles [36]. When the procedure was performed in the presence of a high concentration of cytochrome *c* in the medium, cytochrome *c* was entrapped within the vesicles [37]. Similarly as in intact heart mitochondria, the release of cytochrome *c* from such vesicles was significantly increased by Bax $\Delta$ C, and this effect was partly, though not significantly, prevented by equimolar Bcl-2 (Fig. 2A, left-hand panel).

Outer membrane vesicles could also be filled with commercially available rabbit muscle adenylate kinase (see Section 2). Bax $\Delta$ C did not potentiate the release of adenylate kinase from such vesicles, and no effect was exerted by Bcl-2, either (Fig. 2B).

Some preparations of the outer membrane vesicles were obtained in the presence of 30% glycerol in order to disrupt the contact sites between the outer and the inner mitochondrial membranes as described previously [38]. In fact, the content of succinate dehydrogenase, a marker enzyme for the inner membrane and its remnants in contact sites, was 10 times lower in the outer membrane fraction isolated in the presence

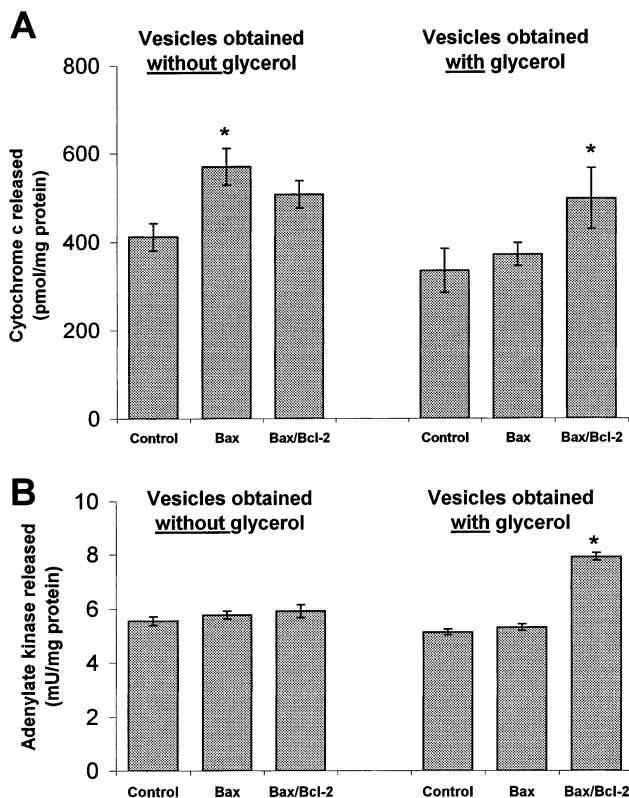


Fig. 2. Effect of Bax $\Delta$ C on the release of cytochrome *c* (A) and adenylate kinase (B) from outer membrane vesicles. Outer membrane vesicles were obtained in the absence or presence of 30% glycerol and loaded with either cytochrome *c* or adenylate kinase as described under Section 2. Release of the entrapped proteins was measured after incubation of the vesicles at 25°C for 30 min in the medium containing 180 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, and 10 mM Tris–HCl (pH 7.4). 1  $\mu$ M Bax $\Delta$ C and 1  $\mu$ M Bcl-2 were added where indicated. The amount of cytochrome *c* entrapped within the vesicles (determined after solubilization with Triton X-100) amounted to  $5000 \pm 100$  and  $4700 \pm 60$  pmol/mg protein for vesicles obtained in the absence and in the presence of glycerol, respectively. Hence, the release of cytochrome *c* from the vesicles prepared in the absence of glycerol (i.e. the difference between the columns designated as ‘Bax’ and ‘Control’) was about 3% of the total amount of cytochrome *c* entrapped. \*Statistically significant difference ( $P < 0.02$ ) with respect to the control.

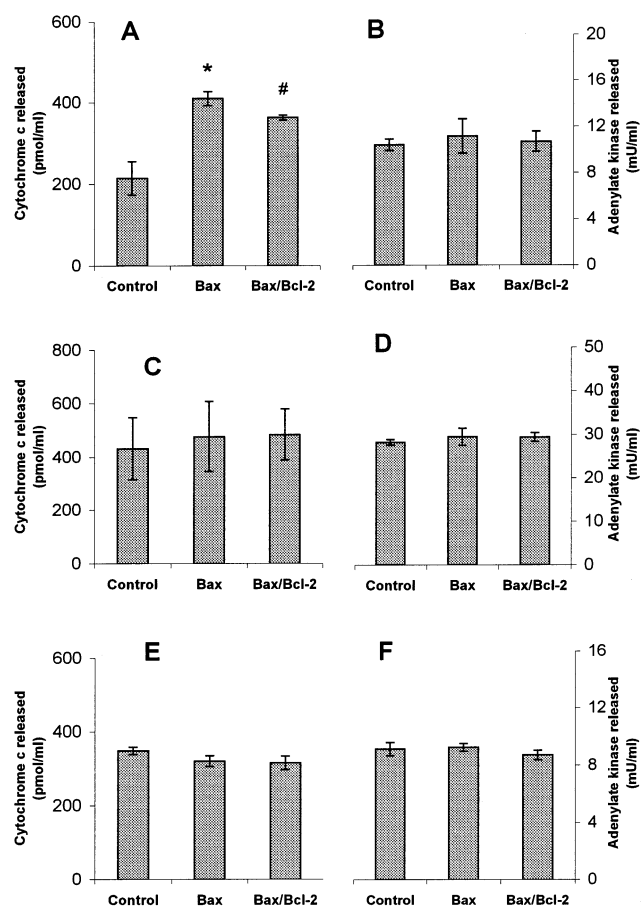


Fig. 3. Effect of Bax $\Delta$ C on the release of cytochrome *c* and adenylate kinase entrapped within phospholipid vesicles reconstituted with the hexokinase-porin-ANT complex or with porin alone or from liposomes containing no reconstituted proteins. The vesicles loaded with cytochrome *c* or adenylate kinase were incubated in 180 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub> and 20 mM HEPES (pH 7.4) buffer for 30 min at 25°C. Bax $\Delta$ C and Bcl-2, 0.74  $\mu$ M each, were present where indicated. A and B, proteoliposomes reconstituted with the hexokinase-porin-ANT complex; C and D, proteoliposomes reconstituted with porin alone; E and F, liposomes without reconstituted protein. The total amount of cytochrome *c* entrapped in the vesicles was  $3100 \pm 900$  pmol/ml in A and  $3450 \pm 450$  in C and  $3100 \pm 200$  in E, and that of adenylate kinase  $100 \pm 5$  mU/ml in B, and  $180 \pm 6$  mU/ml in D and F. \*Statistically significant difference ( $P < 0.005$ ) with respect to the control; #statistically significant difference ( $P < 0.05$ ) with respect to Bax $\Delta$ C.

of glycerol than in the outer membrane fractions isolated without glycerol. It was found that the release of cytochrome *c* from such vesicles deficient in the contact sites was not increased by Bax $\Delta$ C (Fig. 2A, right-hand panel). Surprisingly, Bcl-2 alone (not shown) or in combination with Bax $\Delta$ C (Fig. 2) caused some release of both cytochrome *c* and adenylate kinase from the vesicles deficient in contact sites (obtained in the presence of glycerol). We have no explanation for this peculiar behavior. As it is not cytochrome *c*-specific but also applies to adenylate kinase, it might be related to pore-forming abilities of Bcl-2 observed in artificial phospholipid membranes at acidic pH [42,43].

### 3.2. Bax $\Delta$ C releases cytochrome *c* from liposomes reconstituted with the hexokinase-porin-ANT complex but not from those reconstituted with porin alone

Phospholipid liposomes were reconstituted with the hexokinase-porin-ANT complex from brain tissue and loaded with cytochrome *c* or adenylate kinase as described under Section 2. From such vesicles, Bax $\Delta$ C partly liberated entrapped cytochrome *c* but not adenylate kinase (Fig. 3A,B). The liberation of cytochrome *c* by Bax $\Delta$ C was partly inhibited by Bcl-2 applied at a 1:1 proportion to Bax $\Delta$ C. In contrast, neither cytochrome *c* nor adenylate kinase could be released from phospholipid vesicles reconstituted with porin alone (Fig. 3C,D). Such vesicles were leaky to malate (not shown), indicating that porin was reconstituted in the functionally active form. Phospholipid vesicles containing neither porin nor the hexokinase-porin-ANT complex, filled with cytochrome *c* or adenylate kinase, did not release the entrapped proteins when treated with Bax $\Delta$ C (Fig. 3E,F). These results show that Bax $\Delta$ C does not permeabilize the phospholipid bilayer to cytochrome *c*, nor that the presence of porin alone is sufficient for Bax $\Delta$ C to induce pores large enough to enable cytochrome *c* penetration.

### 3.3. Bax $\Delta$ C increases the accessibility of external cytochrome *c* to the inner mitochondrial membrane in heart and liver mitochondria

It has long been known that freshly isolated mitochondria from liver [35,44], heart muscle [45] and brown adipose tissue [45] oxidize external ferrocytochrome *c* only very slowly. In the present investigation, we showed that external cytochrome *c*, kept reduced by the presence of ascorbate, was oxidized

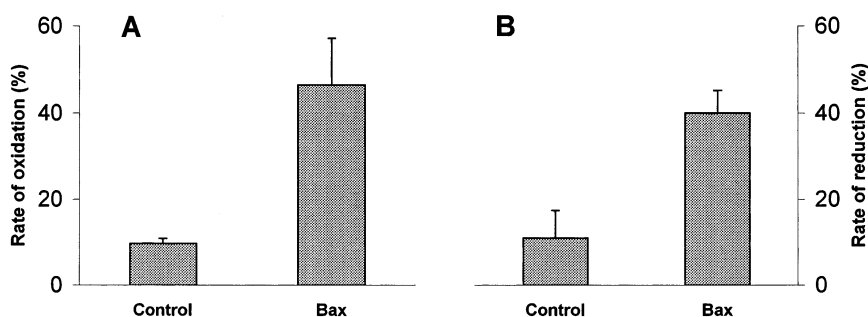


Fig. 4. Oxidation (A) and reduction (B) of external cytochrome *c* by rat liver mitochondria. Mitochondria (1 mg protein/ml in A and 0.33 mg protein/ml in B) were preincubated at 25°C in a medium containing 125 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 0.1 mM cytochrome *c* and additions specified below. The reaction was started by adding 1 mM ascorbate (A, oxidation experiments) or 5 mM succinate (B, reduction experiments). The reaction rate is expressed as percentage of that by mitochondria stripped off the outer membrane by hypotonic swelling or digitonin treatment [46]. Other additions: A, 4  $\mu$ M antimycin A and 0.1 mM 2,4-dinitrophenol; B, 2 mM KCN, 5  $\mu$ M rotenone and 5  $\mu$ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The values in A are means for two mitochondrial preparations and those in B for three preparations  $\pm$  S.D.

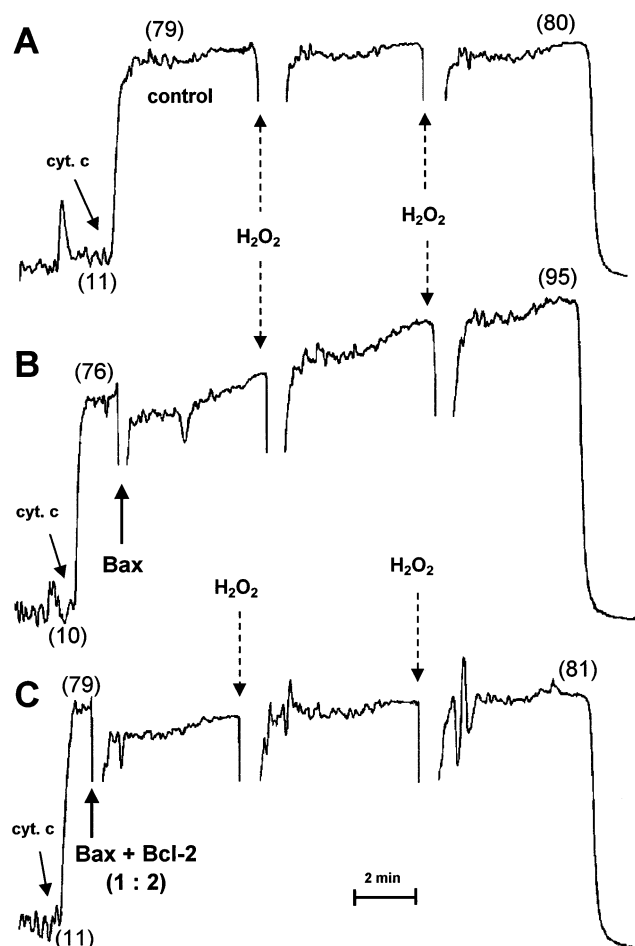


Fig. 5. Oxidation of external cytochrome *c* by rat heart mitochondria. Mitochondria, 1 mg protein, were suspended in 1.0 ml of a medium containing 180 mM KCl, 20 mM Tris-HCl (pH 7.4), 1 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 10 μM antimycin A, 5 μM CCCP, 800 U catalase and 30 μM cytochrome *c*. The traces represent: A, control sample; B, mitochondria to which 6 μM BaxΔC was added; C, mitochondria to which 6 μM BaxΔC and 12 μM Bcl-2 were added where indicated. Small quantities (0.5 mM) of H<sub>2</sub>O<sub>2</sub> were added (dashed arrows) to maintain aerobic conditions. Numbers in parentheses indicate the rate of O<sub>2</sub> uptake in ng atom/min per mg mitochondrial protein. Hypotonically swollen mitochondria (full accessibility of the outer membrane to external cytochrome *c*) respired at the rate of 270 ng atom O/min per mg protein. This figure represents one typical experiment out of three.

very slowly by intact rat liver mitochondria in the medium containing isotonic KCl. Preincubation of the mitochondria for 15 min with micromolar BaxΔC resulted in a several-fold increase of the rate of cytochrome *c* oxidation, amounting to about 50% of the oxidation rate by mitochondria with completely disrupted outer membranes (Fig. 4A).

Fig. 4B shows that the reduction rate of external cytochrome *c* by liver mitochondria was similarly enhanced by preincubation with BaxΔC. In this case electrons were supplied to complex III (via complex II) from succinate and their passage to molecular oxygen was blocked by cyanide.

BaxΔC also increased the rate of oxidation of external cytochrome *c* by rat heart mitochondria (Fig. 5), although the stimulation was much lower than in liver mitochondria. This stimulation was almost completely prevented by Bcl-2 applied in a two-fold excess with respect to BaxΔC (Fig. 5).

#### 4. Discussion

Discussing the results of the present investigation we want to draw attention to the following aspects: (i) specificity of BaxΔC with respect to cytochrome *c*, (ii) what is the primary target for Bax, and (iii) the quantitative aspect of cytochrome *c* release.

##### 4.1. Specificity of BaxΔC effect

The present results (Fig. 1) confirm the findings of other authors [6,8,9,13–15,18,24,47–49] that the proapoptotic protein Bax promotes a release of cytochrome *c* from mitochondria. Whether Bax can trigger the release of adenylate kinase, one of the enzymes present in the intermembrane compartment, has been controversial [16,27–30]. Here we show that BaxΔC does not release adenylate kinase from heart mitochondria under conditions where cytochrome *c* is released. These results strongly oppose earlier assumptions [10–12] that the release of cytochrome *c* and of the AIF at early stages of apoptosis is preceded by disruption of the outer mitochondrial membrane. In addition, we have demonstrated that BaxΔC liberates a part of cytochrome *c* but not adenylate kinase from outer mitochondrial membrane vesicles that contain the contact sites (Fig. 2) and from proteoliposomes reconstituted with the hexokinase–porin–ANT complex (Fig. 3) in which these proteins have been encapsulated. In accordance with the known antagonistic effects of Bax and Bcl-2, the release of cytochrome *c* by BaxΔC was suppressed by Bcl-2. These results are in line with the observations of Doran and Halestrap [16] that endogenous Bax under appropriate conditions released a part of cytochrome *c*, but not of adenylate kinase, from liver mitochondria.

According to a recent report by Gogvadze et al. [30], cytochrome *c* can be released from isolated liver mitochondria by two mechanisms: (i) a Ca<sup>2+</sup>-dependent process, not necessarily connected with large scale mitochondrial swelling, and (ii) a Ca<sup>2+</sup>-independent release enabled by oligomeric Bax. However, as pointed out by the same authors, low amounts of Ca<sup>2+</sup> may induce opening of the permeability transition pore in a small subpopulation of mitochondria that is not depicted by gross swelling and the loss of the membrane potential when measured over the entire population. Hence, a partial release of cytochrome *c* and of the intermembrane isoenzyme of adenylate kinase might be due to disruption of the outer membrane in this small mitochondrial subpopulation. Nevertheless, Gogvadze et al. [30] observed the release of adenylate kinase 2, along with cytochrome *c*, induced by Bax also in the absence of Ca<sup>2+</sup> which is in contrast to our results shown in the present work. This discrepancy may be due to the fact that these authors used full length Bax, whereas in our experiments this proapoptotic protein was deprived of its hydrophobic C-terminal. Both Gogvadze et al. [30] and ourselves used comparable concentrations of Bax. However, the truncated protein BaxΔC applied in our experiments has a much lower affinity to membranes than the full length Bax.

##### 4.2. What is the primary target for Bax in the outer mitochondrial membrane?

Liberation of cytochrome *c* from the outer membrane vesicles by BaxΔC was observed only when the vesicles contained contact sites. Vesicles obtained in the presence of glycerol, a procedure known to disrupt contact sites [38], appeared

not to liberate cytochrome *c* when incubated with Bax $\Delta$ C under identical conditions (Fig. 2). The importance of contact sites for liberation of cytochrome *c* triggered by Bax was confirmed by experiments with phospholipid vesicles reconstituted with the hexokinase–porin–ANT complex, previously shown [39,40] to have properties of native contact sites. Such proteoliposomes, filled with cytochrome *c*, liberated this protein when incubated with Bax $\Delta$ C (Fig. 3A). Proteoliposomes reconstituted with porin alone (Fig. 3C) or liposomes without any reconstituted protein (Fig. 3E) appeared not to become leaky to encapsulated cytochrome *c* in the presence of Bax $\Delta$ C. These results support numerous observations suggesting that Bax does not form cytochrome *c*-conducting pores by itself but, rather, interacts with mitochondrial membrane proteins. There are indications that Bax may form cytochrome *c*-permeable pores in cooperation with porin in the outer membrane [48,50,51] and ANT in the inner membrane [52,53]. Recently, formation of a large non-selective pore that enables the passage of cytochrome *c* across planar phospholipid membranes has been demonstrated by electrophysiological methods for a full length Bax–porin complex [54]. This is, however, in contrast to our results showing that low concentrations of Bax $\Delta$ C produced an outflow of cytochrome *c* only in the presence of the whole hexokinase–porin–ANT complex, thus suggesting the interaction with mitochondrial contact sites [55–57]. This point will be subject to further study.

In accordance with other authors [16,18], we found that Bax enables a bidirectional movement of cytochrome *c* across the outer membrane and does not differentiate between its oxidized and reduced forms.

Our results with protein-free phospholipid vesicles also contrast with the observation of Saito et al. [25] who found liberation of cytochrome *c* from pure phospholipid vesicles by C-terminal truncated Bax. Whether this difference can be contributed to a different Bax protein preparation or a different lipid composition remains unclear. In the experiments of Saito et al. [25] liposomes were formed from a phospholipid mixture containing 30% of the acidic phospholipid, phosphatidic acid, a composition rather unusual for biological membranes.

#### 4.3. Quantitative aspect of cytochrome *c* release by Bax

Several authors (e.g. [18–20,30]) observed a complete release of cytochrome *c* from mitochondria within the cell under apoptotic conditions or from isolated mitochondria under the action of Bax, whereas we found that only a minute part of cytochrome *c* was liberated by low concentrations of Bax $\Delta$ C from isolated mitochondria (Fig. 1, 7.2  $\mu$ M), outer membrane vesicles (Fig. 2, 1  $\mu$ M) and proteoliposomes reconstituted with the hexokinase–porin–ANT complex (Fig. 3, 0.74  $\mu$ M). As discussed above (Section 4.2), the C-terminal truncated Bax used in the present study is not embedded within the membrane, as is the case with full length Bax, and might interact with the membrane by its transmembrane helices 5 and 6. This type of membrane interaction of Bax $\Delta$ C may be primarily targeted to a limited portion of cytochrome *c* that is confined to the contact sites between the outer and the inner membranes. Interaction of cytochrome *c* with porin has already been described by Mannella [58]. In addition, Shimizu et al. [59] have recently reported that antibodies against porin prevent Bax-induced release of cytochrome *c* from isolated mitochondria. Thus, we want to emphasize that a pool of cytochrome *c*, limited in size, is particularly sensitive to the action

of Bax. The low concentration of Bax $\Delta$ C used in these experiments did not form unspecific channels. Yet, it was sufficient to mobilize a specific pool of membrane-associated cytochrome *c*.

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#### References

- [1] Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) *Nature* 397, 441–446.
- [2] Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prévost, M.C., Leber, B., Andrews, D., Penninger, J. and Kroemer, G. (2000) *FASEB J.* 14, 729–739.
- [3] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) *Cell* 86, 147–157.
- [4] Yang, J., Liu, X.S., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J.Y., Peng, T.I., Jones, D.P. and Wang, X.D. (1997) *Science* 275, 1129–1132.
- [5] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) *Science* 275, 1132–1136.
- [6] Tsujimoto, Y. and Shimizu, S. (2000) *FEBS Lett.* 466, 6–10.
- [7] Nechushtan, A., Smith, C.L., Hsu, Y.-T. and Youle, R.J. (1999) *EMBO J.* 18, 2330–2341.
- [8] Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A. and Martinou, J.C. (1998) *J. Cell Biol.* 143, 217–224.
- [9] Jürgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. and Reed, J.C. (1998) *Proc. Natl. Acad. Sci. USA* 95, 4997–5002.
- [10] Scarlett, J.L. and Murphy, M.P. (1997) *FEBS Lett.* 418, 282–286.
- [11] Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T. and Thompson, C.B. (1997) *Cell* 91, 627–637.
- [12] Petit, P.X., Gouben, M., Dolez, P., Susin, S.A. and Kroemer, G. (1998) *FEBS Lett.* 426, 111–116.
- [13] Pastorino, J.G., Tafani, M., Rothman, R.J., Marcinkeviciute, A., Hoek, J.B. and Farber, J.L. (1999) *J. Biol. Chem.* 274, 31734–31739.
- [14] Harris, M.H. and Thompson, C.B. (2000) *Cell Death Differ.* 7, 1182–1191.
- [15] von Ahsen, O., Waterhouse, N.J., Kuwana, T., Newmeyer, D.D. and Green, D.R. (2000) *Cell Death Differ.* 7, 1192–1199.
- [16] Doran, E. and Halestrap, A.P. (2000) *Biochem. J.* 348, 343–350.
- [17] Adachi, S., Gottlieb, R.A. and Babior, B.M. (1998) *J. Biol. Chem.* 273, 19892–19894.
- [18] Kluck, R.M., Degli Esposti, M., Perkins, G., Renken, C., Kuwana, T., Bossy-Wetzel, E., Goldberg, M., Allen, T., Barber, M.J., Green, D.R. and Newmeyer, D.D. (1999) *J. Cell Biol.* 147, 809–822.
- [19] von Ahsen, O., Renken, C., Perkins, G., Kluck, R.M., Bossy-Wetzel, E. and Newmeyer, D.D. (2000) *J. Cell Biol.* 150, 1027–1036.
- [20] Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I. and Green, D.R. (2000) *Nat. Cell Biol.* 2, 156–162.
- [21] Martinou, I., Desagher, S., Eskes, R., Antonsson, B., André, E., Fakan, S. and Martinou, J.-C. (1999) *J. Cell Biol.* 144, 883–889.
- [22] Antonsson, B., Conti, F., Ciavatta, A.M., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.-C., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. and Martinou, J.-C. (1997) *Science* 277, 370–372.
- [23] Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K.A., Hsu, Y.-T., Zimmerberg, J. and Youle, R.J. (1999) *Proc. Natl. Acad. Sci. USA* 96, 5492–5497.

- [24] Appaix, F., Minatchy, M.-N., Riva-Lavieille, C., Olivares, J., Antonsson, B. and Saks, V.A. (2000) *Biochim. Biophys. Acta* 1457, 175–181.
- [25] Saito, M., Korsmeyer, S.J. and Schlesinger, P.H. (2000) *Nat. Cell Biol.* 2, 553–555.
- [26] Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J.-C. (2000) *Biochem. J.* 345, 271–278.
- [27] Single, B., Leist, M. and Nicotera, P. (1998) *Cell Death Differ.* 5, 1001–1003.
- [28] Köhler, C., Gahm, A., Noma, T., Nakazawa, A., Orrenius, S. and Zhivotovsky, B. (1999) *FEBS Lett.* 447, 10–12.
- [29] Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P. and Orrenius, S. (1999) *EMBO J.* 18, 2040–2048.
- [30] Gogvadze, V., Robertson, J.D., Zhivotovsky, B. and Orrenius, S. (2001) *J. Biol. Chem.* 276, 19066–19071.
- [31] Zhai, D., Huang, X., Han, X. and Yang, F. (2000) *FEBS Lett.* 472, 293–296.
- [32] de Pinto, V., Prezioso, G. and Palmieri, F. (1987) *Biochim. Biophys. Acta* 905, 499–502.
- [33] Więckowski, M.R. and Wojtczak, L. (1997) *Biochem. Biophys. Res. Commun.* 232, 414–417.
- [34] Schaller, H., Letko, G. and Kunz, W. (1978) *Acta Biol. Med. Ger.* 37, 31–38.
- [35] Wojtczak, L., Zaluska, H., Wroniszewska, A. and Wojtczak, A.B. (1972) *Acta Biochim. Pol.* 19, 227–234.
- [36] Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) *J. Cell Biol.* 32, 415–438.
- [37] Wojtczak, L. and Sottocasa, G.L. (1972) *J. Membr. Biol.* 7, 313–324.
- [38] Knoll, G. and Brdiczka, D. (1983) *Biochim. Biophys. Acta* 733, 102–110.
- [39] Beutner, G., Rück, A., Riede, B., Welte, W. and Brdiczka, D. (1996) *FEBS Lett.* 396, 189–195.
- [40] Beutner, G., Rück, A., Riede, B. and Brdiczka, D. (1998) *Biochim. Biophys. Acta* 1368, 7–18.
- [41] King, T.E. (1963) *J. Biol. Chem.* 238, 4032–4036.
- [42] Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S. and Montal, M. (1997) *Proc. Natl. Acad. Sci. USA* 94, 5113–5118.
- [43] Schlesinger, P.H., Gross, A., Yin, X.-M., Yamamoto, K., Saito, M., Waksman, G. and Korsmeyer, S.J. (1997) *Proc. Natl. Acad. Sci. USA* 94, 11357–11362.
- [44] Wojtczak, L. and Zaluska, H. (1969) *Biochim. Biophys. Acta* 193, 64–72.
- [45] Zaluska, H., Brabcová, J., Wroniszewska, A., Zborowski, J., Drahota, Z. and Wojtczak, L. (1975) *Exp. Cell Res.* 91, 63–72.
- [46] Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) *J. Cell Biol.* 32, 719–735.
- [47] Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R.J., Matsuda, H. and Tsujimoto, Y. (1998) *Proc. Natl. Acad. Sci. USA* 95, 14681–14686.
- [48] Shimizu, S., Narita, M. and Tsujimoto, Y. (1999) *Nature* 399, 483–487.
- [49] Sawa, H., Kobayashi, T., Mukai, K., Zhang, E. and Shiku, H. (2000) *Int. J. Oncol.* 16, 745–749.
- [50] Shimizu, S., Konishi, A., Kodama, T. and Tsujimoto, Y. (2000) *Proc. Natl. Acad. Sci. USA* 97, 3100–3105.
- [51] Tsujimoto, Y. and Shimizu, S. (2000) *Cell Death Differ.* 7, 1174–1181.
- [52] Marzo, I., Brenner, C., Zamzami, N., Jürgensmeier, J.M., Susin, S.A., Vieira, H.L., Prévost, M.C., Xiem, Z., Matsuyama, S., Reed, J.C. and Kroemer, G. (1998) *Science* 281, 2027–2031.
- [53] Vieira, H.L.A., Haouzi, D., Jacotot, E., Belzacq, A.-S., Brenner, C. and Kroemer, G. (2000) *Cell Death Differ.* 7, 1146–1154.
- [54] Shimizu, S., Ide, T., Yanagida, T. and Tsujimoto, Y. (2000) *J. Biol. Chem.* 275, 12321–12325.
- [55] Brdiczka, D. (1991) *Biochim. Biophys. Acta* 1071, 291–312.
- [56] Brdiczka, D., Beutner, G., Rück, A., Dolder, M. and Wallimann, T. (1998) *BioFactors* 8, 235–242.
- [57] Bühler, S., Michels, J., Wendt, S., Rück, A., Brdiczka, D., Welte, W. and Przybylski, M. (1998) *Proteins* 2, 63–73.
- [58] Mannella, C.A. (1998) *J. Struct. Biol.* 121, 207–218.
- [59] Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y. and Tsujimoto, Y. (2001) *J. Cell Biol.* 152, 237–250.