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Preprotein Transport Machineries of Yeast Mitochondrial Outer Membrane Are not Required for Bax-induced Release of Intermembrane Space Proteins

Luiza K. Sanjuán Szklarz[1], Vera Kozjak-Pavlovic[1], F.-Nora Vögtle[1] Agnieszka Chacinska[1], Dusanka Milenkovic[1], Sandra Vogel[2] Mark Dürr[3], Benedikt Westermann[3], Bernard Guiard[4] Jean-Claude Martinou[5], Christoph Borner[2], Nikolaus Pfanner[1]* and Chris Meisinger[1]

[1]*Institut für Biochemie und Molekularbiologie, Zentrum für Biochemie und Molekulare Zellforschung, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg Germany*

[2]*Institut für Molekulare Medizin und Zellforschung Zentrum für Biochemie und Molekulare Zellforschung Universität Freiburg Stefan-Meier-Straße 17 D-79104 Freiburg, Germany*

[3]*Institut für Zellbiologie Universität Bayreuth, D-95440 Bayreuth, Germany*

[4]*Centre de Génétique Moléculaire, Laboratoire propre du CNRS, 91190 Gif-sur-Yvette France*

[5]*Departement de Biologie Cellulaire, University of Geneva, 1211 Geneva Switzerland*

**Corresponding author*

The mitochondrial outer membrane contains protein import machineries, the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It has been speculated that TOM or SAM are required for Bax-induced release of intermembrane space (IMS) proteins; however, experimental evidence has been scarce. We used isolated yeast mitochondria as a model system and report that Bax promoted an efficient release of soluble IMS proteins while preproteins were still imported, excluding an unspecific damage of mitochondria. Removal of import receptors by protease treatment did not inhibit the release of IMS proteins by Bax. Yeast mutants of each Tom receptor and the Tom40 channel were not impaired in Bax-induced protein release. We analyzed a large collection of mutants of mitochondrial outer membrane proteins, including SAM, fusion and fission components, but none of these components was required for Bax-induced protein release. The released proteins included complexes up to a size of 230 kDa. We conclude that Bax promotes efficient release of IMS proteins through the outer membrane of yeast mitochondria while the inner membrane remains intact. Inactivation of the known protein import and sorting machineries of the outer membrane does not impair the function of Bax at the mitochondria.

Keywords: Saccharomyces cerevisiae; protein import; preprotein translocase; apoptosis; programmed cell death

Present address: V. Kozjak-Pavlovic, Max-Planck-Institut für Infektionsbiologie, Schumannstraße 21/22, D-10117 Berlin, Germany.

Abbreviations used: CCHL, cytochrome *c* heme lyase; $\Delta\psi$, membrane potential; IMS, intermembrane space; Mcr1, mitochondrial NADH-cytochrome b_5 reductase; MIA, mitochondrial intermembrane space import and assembly; PVDF, polyvinylidene difluoride; SAM, sorting and assembly machinery of outer mitochondrial membrane; TIM, translocase of inner mitochondrial membrane; TOM, translocase of outer mitochondrial membrane.

E-mail address of the corresponding author: nikolaus.pfanner@biochemie.uni-freiburg.de

Introduction

Mitochondria consist of four compartments, outer membrane, intermembrane space (IMS), inner membrane and matrix. Though the majority of mitochondrial proteins are located in the inner membrane and matrix, the outer membrane and IMS play crucial roles in various cellular processes. The outer membrane contains the translocase of the outer membrane (TOM complex) that forms the central entry gate for nuclear-encoded precursor proteins, the sorting and assembly machinery (SAM complex) for β-barrel proteins, porins that form pores for metabolites, and numerous components required for fusion, fission and maintenance of mitochondrial morphology.[1–7] The IMS contains machineries for import and assembly of proteins and is involved in the bioenergetic activity of mitochondria and the biogenesis of Fe/S-clusters.[8–14]

Mitochondria play a crucial role in programmed cell death in metazoans. They are the target of various members of the Bcl-2 family, including pro-apoptotic (e.g. Bax, Bak and Bid) and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-X_L).[15–19] The Bcl-2 family members regulate the release of IMS proteins, including cytochrome *c*, from mitochondria and thus control critical steps in apoptosis. Bax promotes a permeabilization of the mitochondrial outer membrane, leading to a release of IMS proteins, while Bcl-2 inhibits this release.[15,16,20]

In recent years, a lower eukaryotic model system has received considerable attention for studying processes of programmed cell death. Cells of the yeast *Saccharomyces cerevisiae* can show characteristics similar to apoptotic mammalian cells, including morphological changes, externalization of phosphatidylserine on the plasma membrane, DNA degradation, condensation of chromatin and release of cytochrome *c* from the mitochondrial IMS.[21–25] A metacaspase (YCA1) has been identified that facilitates cell death in yeast.[26] Although yeast lacks homologues of the Bcl-2 family, expression of mammalian Bax can induce apoptosis-like phenotypes in yeast, including release of cytochrome *c*.[27–33] These effects could be antagonized by co-expression of anti-apoptotic Bcl-2 members.[27,29,30,33]

Different views have been reported on one of the central steps in induction of apoptosis, the mechanism of how Bax mediates release of mitochondrial IMS proteins.[34–39] Initial reports on the involvement of porin (VDAC) in the formation of a Bax-pore[40–42] were revised later in that at least the major isoform (VDAC1) was not required for release of IMS proteins.[32,43] A protease-sensitive component of mitochondria was implicated in Bid-induced oligomerization of Bax but not characterized further.[43] Another study, however, showed that Bid-activated Bax produced membrane openings in the absence of other proteins.[44] Moreover, a connection between mitochondrial outer membrane permeabilization and the machinery regulating mitochondrial fission was proposed for both mammalian cells and yeast.[5,24,45–47] It has been speculated that the TOM and/or SAM machineries may be involved in pore formation in the outer mitochondrial membrane as a key step in apoptosis;[1,18,48–50] however, experimental evidence has been scarce.

To study a role of endogenous mitochondrial proteins in Bax-mediated release of IMS proteins, we characterized the release from isolated yeast mitochondria. With a large collection of mutants deficient in outer membrane proteins we demonstrate that Bax induces release of IMS proteins independently of the activity of all TOM, SAM, fusion, and fission proteins analyzed. Protein complexes up to a size of 230 kDa were released from yeast mitochondria upon addition of Bax while the inner membrane barrier and function remained intact, pointing to a selective but massive release of IMS proteins mediated by Bax itself.

Results

Bax-induced release of cytochrome *c* from yeast mitochondria does not depend on protease-sensitive outer membrane proteins

We incubated isolated yeast mitochondria with recombinant full-length human Bax that was prepared in the monomeric form (Figure 1(a)).[32,40,41,43,51] Bax promoted an efficient release of cytochrome *c* to the supernatant while the matrix heat shock protein Hsp60 remained inside the mitochondria (Figure 1(b)). The majority of cytochrome *c* was released after an incubation of 20 min (Figure 1(c)). Similarly, the IMS protein Tim10 (a translocase subunit of about 10 kDa) was released after a 20 min incubation of mitochondria with Bax (Figure 1(c)).

Upon treatment of the mitochondria at alkaline pH (sodium carbonate), the majority of Bax was not extracted (Figure 1(d)), indicating that a major fraction of mitochondria-associated Bax was integrated into the membranes, like the outer membrane protein porin. The control protein Hsp60 was efficiently extracted by carbonate treatment (Figure 1(d)). Blue native electrophoresis showed that mitochondria-associated Bax formed larger oligomeric forms in a time-dependent manner (Figure 1(e)). To determine if the Bax-treated yeast mitochondria contained an intact, active inner membrane, we analyzed the import of a radiolabeled precursor protein into the matrix.[52,53] F_1-ATPase subunit β was efficiently imported in the presence and absence of Bax (Figure 1(f), lanes 2–5 and 7–10) while dissipation of the membrane potential ($\Delta\psi$) by the potassium ionophore valinomycin blocked protein import under both conditions (Figure 1(f), lanes 1 and 6). Thus, similar to the findings with mitochondria from higher eukaryotes,[54] the yeast mitochondrial inner membrane remains active for protein import despite the efficient release of cytochrome *c* upon addition of Bax. Protein translocation across the inner membrane can bypass the

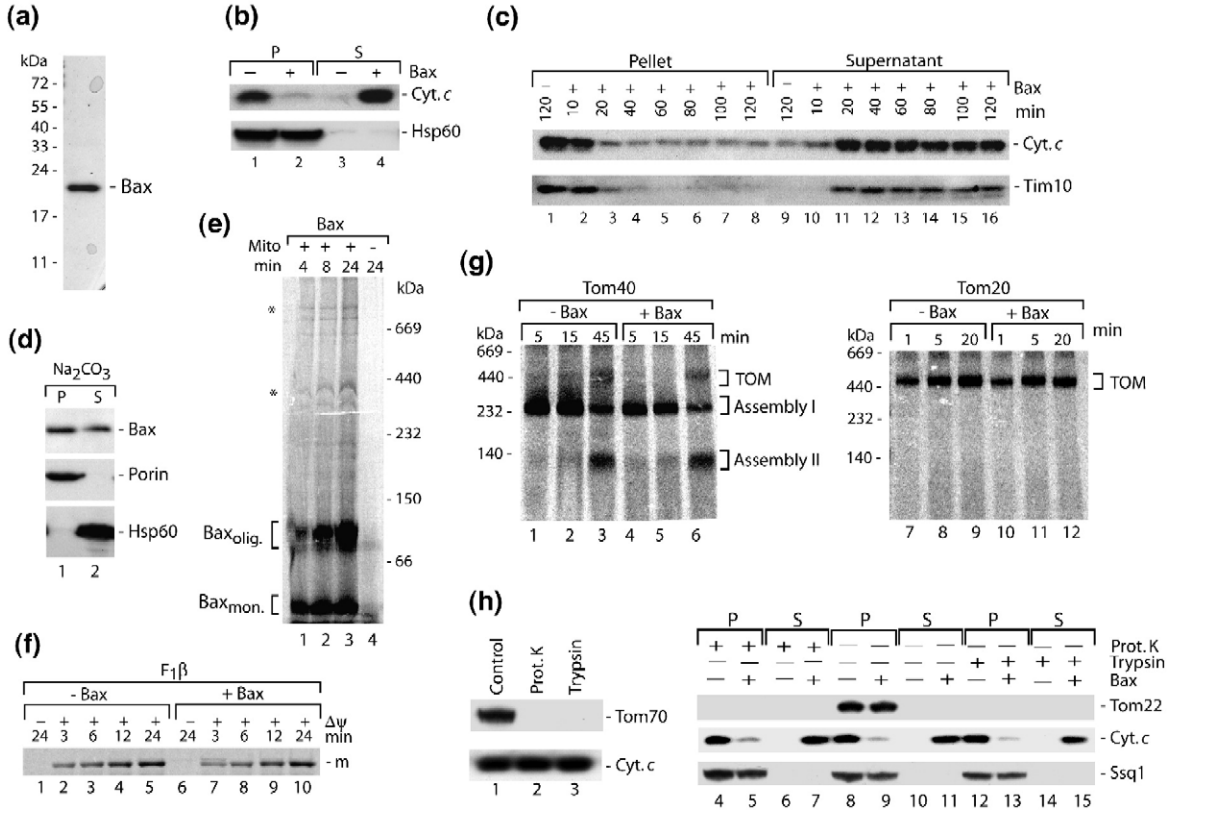

Figure 1. Bax induces release of cytochrome *c* from yeast mitochondria independently of surface receptors and does not block protein import. (a) Purified recombinant Bax protein was separated by SDS–PAGE and stained with Coomassie brilliant blue. (b) Isolated yeast wild-type mitochondria were incubated in the presence or absence of Bax as described in Materials and Methods. After 1 h of incubation the samples were subjected to centrifugation and the mitochondrial fraction (pellet, P) was separated from the supernatant (S). Cytochrome *c* and the matrix protein Hsp60 were detected *via* Western blot analysis. (c) Mitochondria were incubated with Bax for different periods and analyzed for release of IMS proteins by Western blotting. The release assay was stopped by centrifugation and immediate separation of the mitochondrial fraction (pellet) from the supernatant. (d) Mitochondria were incubated with Bax for 20 min and treated with sodium carbonate (pH 11.5). Pellet and supernatant were separated and analyzed by SDS–PAGE and Western blotting. For detection of Bax, a commercial monoclonal anti-human Bax antibody (Sigma-Aldrich) was used. (e) ^{35}S-labeled Bax was imported into mitochondria for the indicated periods (min). The mitochondria were pelleted by centrifugation, lysed by digitonin and analyzed by blue native electrophoresis. As mock control, the reaction was performed in the absence of mitochondria (lane 4). Bax was detected by digital autoradiography. Asterisks, high molecular mass forms of Bax imported into mitochondria. (f) Import of the ^{35}S-labeled matrix protein $F_1\beta$ into mitochondria, which were incubated in the presence or absence of Bax prior to the import reaction. After proteinase K treatment and SDS–PAGE, imported mature (m) protein was visualized by digital autoradiography. (g) Import of ^{35}S-labeled outer membrane proteins, Tom20 and Tom40, into mitochondria incubated in the presence or absence of Bax prior to the import reaction. After lysis in digitonin buffer and blue native electrophoresis, imported and assembled proteins were visualized by digital autoradiography. (h) Mitochondria were pretreated with proteases prior to their incubation in the presence or absence of Bax. The release of cytochrome *c* and control proteins was analyzed by Western blotting. P, pellet/mitochondrial fraction; S, supernatant; Prot. K, proteinase K.

requirement for the outer membrane, i.e. it is also possible in mitochondria with ruptured outer membrane.[55,56] We thus asked if protein assembly in the outer membrane was blocked by Bax treatment. By blue native electrophoresis, we monitored the assembly pathways of the radiolabeled precursors of Tom40 and Tom20 of the TOM complex.[57–60] Remarkably, both precursors could still be imported into the outer membrane; the treatment with Bax only moderately impaired the efficiency of Tom40 assembly (Figure 1(g)). Since rupturing of the outer membrane by swelling of mitochondria completely blocks the assembly pathway of Tom40,[59] it is evident that Bax-treated mitochondria maintain critical functions of the outer membrane and do not simply reflect swollen mitochondria with a ruptured outer membrane. Electron microscopic analysis confirmed that the Bax-treated mitochondria were not swollen (not shown).

We then asked if the transfer of Bax to mitochondria and its activity in cytochrome *c* release depended on mitochondrial surface proteins. The isolated yeast mitochondria were pretreated with two different proteases, trypsin and proteinase K, which are known to remove all cytosolic domains of

the Tom receptors,[52,61] shown here for Tom70 (Figure 1(h), lanes 2 and 3) and Tom22 (Figure 1(h), lanes 4, 5, 12 and 13). Then Bax was added yet the release of cytochrome *c* was not impaired (Figure 1(h), lanes 7 and 15) and occurred with similar efficiency as in non-treated mitochondria (Figure 1(h), lane 11). As control we used the matrix heat shock protein Ssq1 that remained fully in the mitochondrial fraction. We conclude that the receptor domains of the Tom receptors are not required for the transport of Bax to mitochondria and the induction of cytochrome *c* release.

Tom proteins are not required for Bax-induced release of cytochrome *c*

Different views have been reported on a possible role of the major porin isoform, Por1 (VDAC1) in Bax-induced cytochrome *c* release.[32,40,41,43] Mitochondria isolated from *por1*Δ yeast efficiently released cytochrome *c* upon incubation with Bax (Figure 2(a), lane 4) in agreement with the results reported by Roucou *et al.*[43] and Priault *et al.*[32] Tim10 was similarly released, while the peripheral inner membrane protein cytochrome *c* heme lyase (CCHL) was retained in the mitochondria (Figure 2(a), lane 2). Yeast contains a second porin isoform of lower abundance, Por2,[62] which has not been analyzed so far for a possible role in Bax-mediated release of IMS proteins. *por2*Δ mitochondria were not impaired in release of cytochrome *c* and Tim10 (Figure 2(a)), indicating that the activity of Bax did not require Por2. We also studied a *por1*Δ *por2*Δ double mutant; however, these mutant cells grow only poorly[62] and we observed that the isolated mitochondria contained a damaged ("leaky") outer membrane and released IMS proteins independently of the presence of Bax (not shown).

The essential subunit of the TOM complex, Tom40, forms a channel through the outer membrane.[63–65] We analyzed Bax-induced release of cytochrome *c* in three different mutants of Tom40[18,66,67] but did not detect a significant impairment of release (Figure 2(b), lane 4). The TOM complex contains three receptors, Tom20, Tom22 and Tom70, and small Tom proteins. Mitochondria lacking Tom20 or Tom70, as well as mitochondria lacking Tom7, efficiently released cytochrome *c* upon incubation with Bax (Figure 2(b), lane 8). Deletion of the third receptor, Tom22, leads to a loss of mitochondrial DNA (rho^0).[68] It has been shown that in *tom22*Δ mitochondria, the steady-state levels of cytochrome *c* are reduced compared to rho^0 wild-type mitochondria independently of the presence of Bax.[69] We asked if Tom22 was required for the action of Bax at mitochondria, yet the remaining cytochrome *c* was efficiently released from *tom22*Δ mitochondria (Figure 2(c)). A quantification of release based on the total amount of cytochrome *c* present in the respective mitochondria revealed that the kinetics of release were not reduced in *tom22*Δ mitochondria compared to wild-type mitochondria (Figure 2(d)).

Thus, the results with Tom receptor deletion mutants agree with the experiments shown in Figure 1(h), where the receptor domains were

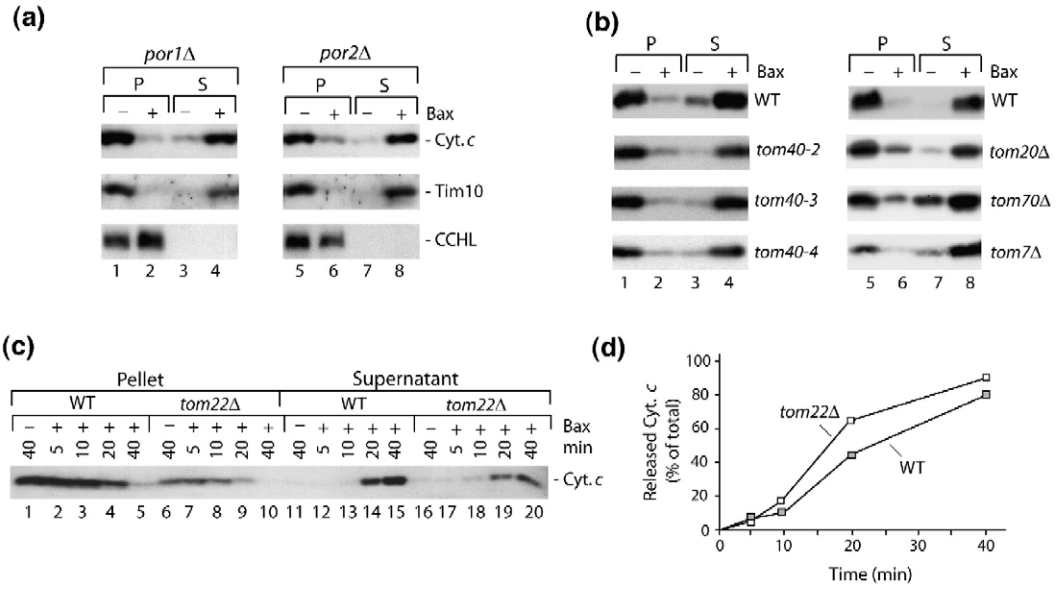

Figure 2. Yeast mitochondria defective in porins or Tom proteins are not affected in Bax-induced release of cytochrome *c*. (a) Incubation of *por1* and *por2* deletion mitochondria[62] in the absence or presence of Bax was performed as described in Materials and Methods. Pellet (P) and supernatant (S) fractions were separated by SDS–PAGE and analyzed by Western blotting using antisera against the indicated proteins. (b) Analysis of cytochrome *c* release upon incubation of mutant mitochondria of Tom proteins with Bax.[66,67] WT, wild-type. (c) Analysis of cytochrome *c* release at different time points in *tom22*Δ mitochondria and the corresponding rho^0 wild-type mitochondria.[68] (d) Quantitive analysis of cytochrome *c* release in *tom22*Δ mitochondria compared to rho^0 wild-type mitochondria.

removed by protease and the activity of Bax in protein release was not impaired. We conclude that Bax induces efficient release of cytochrome *c* independently of the inactivation or deletion of Tom proteins.

Inactivation of the SAM complex does not inhibit Bax-induced release of cytochrome *c* from mitochondria

The recent identification of a second protein translocase in the mitochondrial outer membrane, the SAM complex, promoted the suggestion that this translocase may be required for release of IMS proteins during apoptosis.[1,48–50] We asked if Bax required SAM components for permeabilization of the mitochondrial outer membrane. Since two SAM subunits are essential for cell viability we generated conditional mutants[50,70] and selected three mutants of each, Sam35 and Sam50, for the *in organello* release assay. Bax promoted an efficient release of cytochrome *c* in the *sam35* and *sam50* mutants similar like in wild-type mitochondria (Figure 3). Moreover, mitochondria isolated from deletion mutants of the two further SAM subunits, Sam37 and Mdm10, released cytochrome *c* upon incubation with Bax (Figure 3). Since *mdm10*Δ cells show a high tendency to lose mitochondrial DNA,[71,72] we used rho^0 mitochondria as control.[60] We conclude that the activity of the SAM complex is not critical for the action of Bax at mitochondria.

Cytochrome *c* release is not impaired by inactivation of mitochondrial fission and fusion machineries

It has been reported that components of the mitochondrial fission machinery are involved in regulation of programmed cell death.[5,24,45–47] We thus analyzed Bax-induced release of cytochrome *c* from mitochondria lacking various subunits of the fission (Dnm1, Fis1 and Mdv1) and fusion machineries (Fzo1 and Ugo1). Cytochrome *c* was efficiently released, indicating that these proteins were not critical for Bax-mediated release (Figure 4(a)).

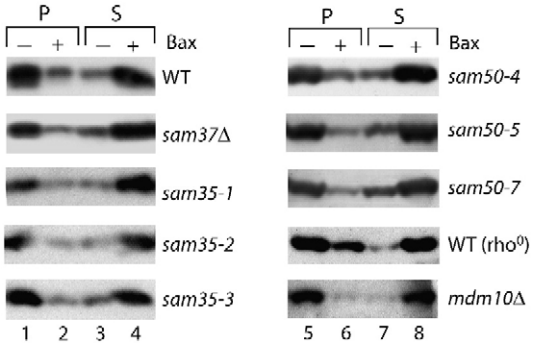

Figure 3. Yeast mutants of the SAM complex are not impaired in Bax-induced release of cytochrome *c*. Mutant mitochondria of *sam37*,[58] *sam35*,[50] *sam50*[70] and *mdm10*[60] were incubated in the presence or absence of Bax as described in Materials and Methods. The efficiency of cytochrome *c* release was determined by Western blot analysis. P, pellet/mitochondrial fraction; S, supernatant.

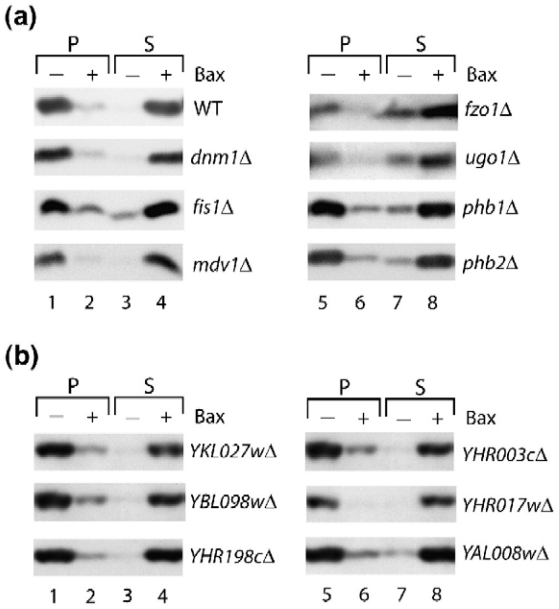

Figure 4. Bax-induced release of cytochrome *c* in mutants of proteins regulating mitochondrial morphology and novel outer membrane proteins. (a) Yeast mutant mitochondria (all from Euroscarf collection except *phb1*Δ and *phb2*Δ, which were generated in the YPH499 background[81]) were incubated in the presence or absence of Bax as described in Materials and Methods. Cytochrome *c* release was determined by Western blot analysis. WT, wild-type. (b) Cytochrome *c* release upon Bax treatment of mutant mitochondria (Euroscarf collection) of novel proteins identified in the mitochondrial outer membrane proteome.[7]

Prohibitins 1 and 2 are mitochondrial inner membrane proteins with large domains facing the IMS.[73–75] The exact molecular function of prohibitins is unknown yet an involvement in regulation of mitochondrial morphology[76] and apoptosis has been reported.[77,78] Mitochondria, which were isolated from yeast mutants lacking the genes for prohibitin 1 or 2, efficiently released cytochrome *c* upon incubation with Bax (Figure 4(a)).

Moreover, we tested several new mitochondrial outer membrane proteins that were identified in a recent proteomic study.[7] Mitochondria from yeast deletion mutants of those proteins were not impaired in Bax-induced protein release (Figure 4(b)). Thus, none of the mitochondrial components analyzed was required for Bax-mediated release of cytochrome *c* from isolated yeast mitochondria.

Bax induces release of large protein complexes from the mitochondrial intermembrane space

To analyze the specificity of Bax-mediated protein release, we probed for several different proteins in the release assay with wild-type yeast mitochondria. In addition to the small IMS proteins

cytochrome *c* (12 kDa), Tim10 (10 kDa) and Tim13 (11 kDa), the IMS proteins Erv1 (22 kDa), $Mcr1_{IMS}$ (30 kDa) and cytochrome b_2 (57 kDa) were efficiently released in the presence of Bax (Figure 5(a)). The outer membrane form of Mcr1[79] as well as the outer membrane proteins Tom40, Tom70 and porin were not released (Figure 5(a)), indicating that the outer membrane components remained associated with the mitochondria. Similarly, IMS proteins attached to the inner membrane, such as CCHL and Mia40, stayed in the mitochondrial pellet. The matrix heat shock proteins Ssc1 and Ssq1 also stayed in the mitochondrial pellet (Figure 5(a)), indicating that the inner membrane remained intact. Thus, all soluble IMS proteins analyzed were released by addition of Bax, the largest one being cytochrome b_2 with a monomeric molecular mass of 57 kDa.[80]

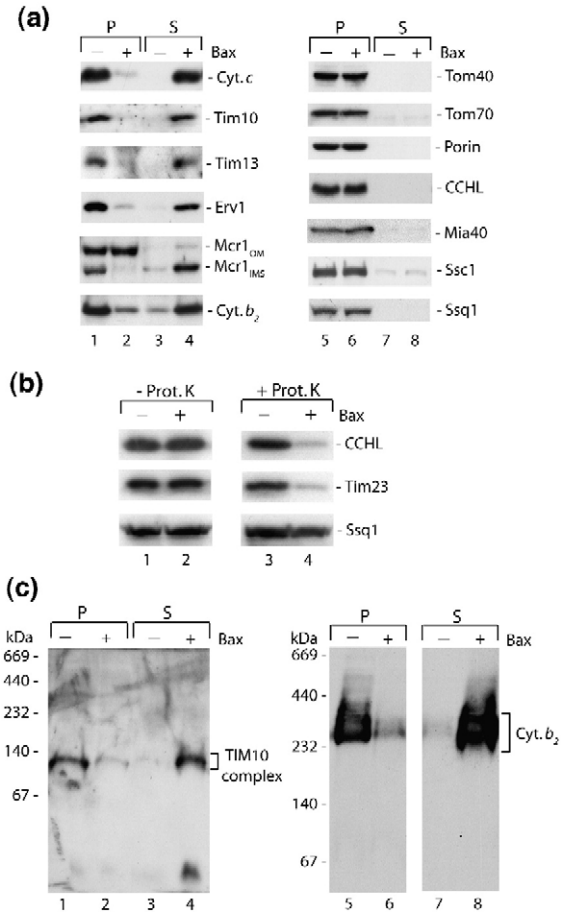

Figure 5. Bax induces release of large protein complexes from the mitochondrial IMS. (a) Yeast wild-type mitochondria were incubated in the presence and absence of Bax. Mitochondrial proteins of the IMS, matrix and outer membrane (OM) were analyzed by Western blot analysis. P, pellet/mitochondrial fraction; S, supernatant. Erv1, sulfhydryl oxidase (IMS); Mcr1, NADH-cytochrome b_5 reductase; Cyt. b_2, cytochrome b_2 (IMS); Ssc1 and Ssq1, Hsp70 proteins of the matrix. (b) Mitochondria were incubated in the presence or absence of Bax, re-isolated and treated with proteinase K (50 μg/ml) for 15 min on ice. Accessibility of mitochondrial IMS proteins to the protease was determined by SDS–PAGE and Western blot analysis. (c) Upon incubation of mitochondria in the presence or absence of Bax, the supernatant (S) and mitochondrial (P) fractions were analyzed by blue native electrophoresis and Western blotting as described in Materials and Methods.

When Bax-treated mitochondria were incubated with proteinase K, proteins exposed to the IMS were degraded, including CCHL and Tim23 (Figure 5(b)). Ssq1 of the matrix was protected against the added protease (Figure 5(b)). Thus, upon Bax treatment, mitochondria do not only release proteins but a protease with a molecular mass of 29 kDa can enter the IMS.

Some IMS proteins, which were released upon Bax treatment (Figure 5(a)), are present in oligomeric complexes in mitochondria. We asked whether these proteins were released to the supernatant as monomers or oligomers. Tim10 and Tim9 form a hexameric complex of 60 kDa ($Tim10_3$–$Tim9_3$) that can be visualized by blue native electrophoresis.[81,82] Indeed, Tim10 released to the supernatant migrated predominantly in the same range on blue native gels as the mitochondrial hexameric complex (Figure 5(c), lanes 1 and 4). Arnoult *et al.*[47] reported that the Bax/Bak-dependent release of human DDP/TIMM8a, a homolog of the small Tim proteins of the yeast mitochondrial IMS, promoted Drp1-mediated mitochondrial fragmentation. Our observation suggests that the small Tim proteins are released as oligomeric complexes and thus may function as oligomers in the cytosol. Cytochrome b_2 forms a homotetramer of 230 kDa.[83] Remarkably the entire tetramer was almost quantitatively released to the supernatant upon Bax treatment as determined by blue native electrophoresis (Figure 5(c), lane 8). The cytochrome b_2 tetramer is a stable complex,[83,84] indicating that the entire complex can pass through the outer membrane.

Our studies shown in Figures 1–4 did not reveal a dependence on an endogenous mitochondrial protein for the Bax-induced release of the 12 kDa protein cytochrome *c*. We wondered if the release of a large protein such as cytochrome b_2 was also independent of endogenous mitochondrial proteins. In a first approach, we pretreated wild-type mitochondria with either proteinase K or trypsin to remove the surface receptors. After addition of Bax, cytochrome b_2 was efficiently released (Figure 6(a), lanes 4 and 12), indicating that the Tom receptors were not needed for the action of Bax. We confirmed this with mutant mitochondria lacking Tom20 and Tom70 (Figure 6(b), lane 4). Similarly, three different mutant mitochondria of the channel Tom40, as well as *tom7*Δ mitochondria were not impaired in Bax-induced release of cytochrome b_2 (Figure 6(b), lane 4). All further mutant mitochondria analyzed, including those for Sam35, Sam37 and Sam50, as well as the fission components Dnm1 and Mdv1, released cytochrome b_2 upon incubation with Bax (Figure 6(b), lane 8).

We conclude that Bax induces the release of large protein complexes from the IMS of yeast

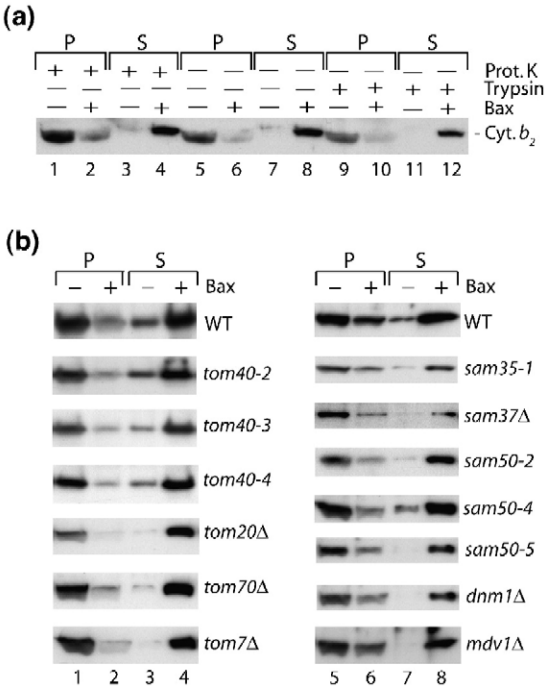

Figure 6. Bax-induced release of cytochrome b_2 from the mitochondrial IMS does not depend on TOM and SAM complexes. (a) Wild-type mitochondria were pretreated with proteases as described in Materials and Methods before incubation in the presence or absence of Bax. Cytochrome b_2 release was analyzed by SDS–PAGE and Western blotting. (b) Cytochrome b_2 release in different mutant mitochondria. P, pellet/mitochondrial fraction; S, supernatant.

mitochondria without a requirement for the protein transport machineries of the outer membrane.

Discussion

We report that human Bax induces a permeabilization of the outer membrane of yeast mitochondria that leads to the release of all soluble IMS proteins studied, including protein complexes with a size of 230 kDa. This massive release of IMS proteins occurs while the mitochondrial inner membrane remains intact and is competent in import of precursor proteins. The permeabilization of the outer membrane not only permits the export of IMS proteins but also the transport of folded proteins into the IMS, as shown with added proteinase K.

Currently, different views are discussed on how Bax induces a permeabilization of the mitochondrial outer membrane. (i) Bax could allow the entry of water or solutes to the mitochondrial matrix, leading to swelling of mitochondria and disruption of the outer membrane.[32] (ii) Bax forms a channel in the outer membrane without a requirement for endogenous mitochondrial proteins.[44,85] (iii) Channel formation by Bax depends on pre-existing mitochondrial proteins such as porins or outer membrane protein translocases.[40,41,43,48,86,87]

Possibility (i): The outer membrane of Bax-treated mitochondria is not simply ruptured like in swollen mitochondria. We found that the assembly pathway of the precursor of Tom40 was only moderately impaired in those mitochondria under conditions that lead to an efficient release of IMS proteins. A rupturing of the outer membrane by swelling of mitochondria has been shown to completely block the assembly pathway of Tom40[59] and thus this situation is quite different from the behavior of Bax-treated mitochondria. We conclude that Bax induces a selective permeabilization of the outer membrane.

To distinguish between possibilities (ii) and (iii), we made use of a large collection of yeast mutants of mitochondrial outer membrane proteins, including deletion mutants and conditional mutants in case of essential genes. Of particular importance was the question if the TOM complex and/or the SAM complex played a role in Bax-mediated release of IMS proteins. The (weak) similarity of Sam37 to mammalian metaxin 1, which participates in tumor necrosis factor-induced cell death,[88] suggested a possible relation of SAM to outer membrane permeabilization.[49,50] However, neither removal of the receptor binding domains by protease treatment nor mutants of various Tom and Sam proteins impaired the release of IMS proteins, even of the large cytochrome b_2, indicating that Bax can function independently of the two preprotein transport machineries of the outer membrane. We confirmed previous findings that VDAC1 was not required.[32,43] In addition, we show that VDAC2 of yeast mitochondria is not required for Bax-mediated protein release. All further yeast mutants analyzed, including those for mitochondrial fission and fusion components, were competent in the release of IMS proteins upon incubation with Bax. Thus, our findings with the model system *S. cerevisiae* favor the view of Saito *et al.*[85] and Kuwana *et al.*[44] that Bax forms channels in the mitochondrial outer membrane independently of the presence of endogenous mitochondrial proteins. The involvement of mitochondrial fission components in programmed cell death may take place downstream of permeabilization of the outer membrane.[47]

Pavlov *et al.*[87] showed that expression of human Bax in yeast led to the formation of a channel in the mitochondrial outer membrane that was related to the mitochondrial apoptosis-induced channel (MAC) of apoptotic mammalian cells. From electrophysiological studies, a pore diameter of up to 4 nm was assessed.[87] Such a pore would permit the export of globular proteins with a molecular mass up to 20–30 kDa. The pore formed in our *in organello* system with yeast mitochondria and Bax has to be significantly larger as the 230 kDa tetramer of cytochrome b_2 was efficiently released.

In summary, the *in organello* system with isolated yeast mitochondria characterized here suggests that Bax promotes permeabilization of the outer membrane independently of endogenous mitochondrial proteins. Bax-treated mitochondria efficiently release all kinds of soluble IMS proteins, including

large protein complexes. Despite this massive release, the outer membrane is not unspecifically ruptured and the integrity of the inner membrane remains protected in the time course of the experiments. The excellent genetic and biochemical accessibility of yeast thus provides an interesting *in organello* model system to study mitochondria-related steps of programmed cell death.[22,25,89–94]

Materials and Methods

Isolation of mitochondria

S. cerevisiae cells were grown at 20–23 °C in YP medium (1% (w/v) yeast extract, 2% (w/v) Bactopeptone) containing either 2% (w/v) sucrose (YPS) or 3% (w/v) glycerol (YPG) as carbon source. After reaching an optical density between 0.6 and 1.2, cells were harvested, homogenized and mitochondria were isolated by differential centrifugation as described.[95,96] After resuspension in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops–KOH(pH 7.2)), mitochondria were aliquoted and stored at −80 °C.

Assay for Bax-induced release of IMS proteins from isolated mitochondria

Recombinant full-length human Bax with a hexahistidine tag was isolated in monomeric form as described by Roucou *et al.*[43] Mouse tBid was generated by cleavage of recombinant full-length Bid with caspase 8.[97] Release assays were performed essentially as described.[51,98] Briefly, isolated yeast mitochondria (100 μg protein) were resuspended in 100 μl of assay buffer (250 mM sucrose, 150 mM KCl, 10 mM Mops–KOH (pH 7.2)). After addition of 50 nM Bax and 10 nM tBid, samples were incubated for 1 h at 37 °C. The supernatant and mitochondrial fractions were separated by centrifugation at 20,000*g* for 15 min at 4 °C. For Tris-tricine SDS–PAGE,[99] proteins from the supernatant fractions were precipitated with 10% (w/v) trichloroacetic acid or StrataClean Resin (Stratagene). For Western blotting, proteins were transfered onto PVDF membranes and detected by immunodecoration using specific antisera and the Enhanced Chemoluminescence detection system (GE healthcare). Treatment of mitochondria with sodium carbonate was performed as reported.[61,100] Electron microscopic analysis of purified mitochondria was performed as described.[101] The assays typically contained Bax and tBid. In agreement with Roucou *et al.*[43] we observed that tBid itself did not promote release of IMS proteins and full-length Bax alone was efficient in protein release.

Blue native electrophoresis of released complexes

Wild-type mitochondria (0.6 to 1 mg protein) were incubated for 1 h at 37 °C in 100 μl of assay buffer in the presence or absence of Bax/tBid as described above. Fractions of 10 μl were centrifuged at 20,000*g* for 15 min at 4 °C. The pellet containing mitochondria was solubilized in 65 μl of digitonin buffer (1% (w/v) digitonin, 20 mM Tris–HCl (pH 7.4), 10% (v/v) glycerol, 50 mM NaCl, 0.1 mM EDTA). The supernatant was diluted with 65 μl of digitonin buffer. After incubation for 15 min on ice and 15 min centrifugation at 20,000*g*, 5 μl of sample buffer (5% (w/v) Coomassie brilliant blue G250, 500 mM 6-aminocaproic acid, 100 mM bis–Tris (pH 7.0)) was added to the supernatant. After separation on a 6%–16.5% (w/v) polyacrylamide gel, protein complexes were detected by immunodecoration.

Protease treatment of mitochondria

Mitochondria were resuspended in import buffer (10 mM Mops–KOH (pH 7.2), 3% (w/v) bovine serum albumin, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl) and incubated in the presence of either proteinase K (40 μg/ml) or trypsin (40 μg/ml) for 15 min on ice. Protease treatment was stopped by adding 1 mM phenylmethylsulfonyl fluoride or a 30-fold excess of soybean trypsin inhibitor, respectively.

In vitro import of preproteins into Bax-treated mitochondria

Mitochondria were incubated in assay buffer in the presence or absence of Bax/tBid for 20 min at 37 °C. After re-isolation they were resuspended in import buffer (10 mM Mops–KOH (pH 7.2), 3% bovine serum albumin, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl) containing 2 mM NADH, 2 mM ATP, 5 mM creatine phosphate and 100 μg/ml of creatine kinase. Radiolabeled precursor proteins were generated by *in vitro* transcription/translation using rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine (GE healthcare).[52,61] A cDNA of human Bax was obtained from the German Resoure Center for Genome Research. Mitochondria were incubated with precursor proteins in import buffer for various periods at 25 °C. After washing with SEM buffer, samples were either treated with 50 μg/ml proteinase K and subjected to SDS–PAGE or solubilized in 1% digitonin buffer and separated by blue native electrophoresis.[52] Gels were dried and labeled proteins were visualized by PhosphorImaging (GE healthcare).

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