The cytotoxic action of Bax on yeast cells does not require mitochondrial ADP/ATP carrier but may be related to its import to the mitochondria

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Abstract The effect of the expression of murine Bax protein on growth and vitality was examined in *Saccharomyces cerevisiae* and compared with the effect of Bax in mutant cells lacking functional mitochondria. The cytotoxic effect of Bax on yeast does not require functional oxidative phosphorylation, respiration, or mitochondrial proteins (ADP/ATP carriers) implicated in the formation of the permeability transition pore in mammalian mitochondria. In the wild type *S. cerevisiae* the expression of Bax does not result in a severe effect on mitochondrial membrane potential and respiration. On the basis of Bax induced differences in the fluorescence of green fluorescent protein fused to mitochondrial proteins, it is proposed that Bax may interfere with one essential cellular process in yeast: the mitochondrial protein import pathway that is specific for the proteins of the mitochondrial carrier family.

Key words: Apoptosis; ADP/ATP carrier; Bax; Mitochondrion; Yeast

1. Introduction

The finding that the proteins encoded by the genes of the Bcl-2 family interfere with the growth of yeast cells [1–3] has initiated the use of this organism as a tool for investigating the mechanism of action of proteins involved in the metazoan apoptosis. One important aspect of this mechanism is the participation of mitochondria in cell death [4,5]. Mitochondria have been shown to play a key function in the decisive phase of metazoan apoptosis, in which the interaction between mitochondrial membranes and proapoptotic proteins (e.g. Bax and Bak) leads to a release of proteins, such as cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria to the cytosol [6–9]. These events are prevented by anti-apoptotic members of the Bcl-2 family of proteins [7,8,10]. Upon entering the cytosol, cytochrome *c* forms a complex with Apaf1, a homologue of the *Caenorhabditis elegans* protein ced-4, and caspase 9, which further triggers caspase activation and cell death [11].

The release of proteins from mammalian mitochondria probably results from the opening of an unspecific mitochondrial channel termed permeability transition pore (PTP) and this event is either preceded, or accompanied by loss of mitochondrial transmembrane potential ($\Delta\Psi$) [7,8,12,13]. It has been proposed that PTP is a complex of several proteins including inner membrane ADP/ATP carrier (AAC), the outer membrane voltage-dependent anion channel (VDAC), and the matrix cyclophilin D [6,14]. The central role of AAC in PTP and apoptosis is supported by observations that some forms of apoptosis are suppressed by a specific ligand of AAC, bongkrekic acid, that inhibits both mitochondrial ADP/ATP transport and PTP opening [12,15,16]. Evidence was recently presented that Bax and AAC cooperate within PTP [17,18] to increase mitochondrial permeability and to trigger cell death. The failure of Bax to kill AAC-less yeast strain was interpreted to indicate that Bax and AAC cooperate in the mitochondrial control of apoptosis [17].

The goal of understanding the mechanisms that trigger cell death would be facilitated by being able to assess the function of apoptosis-regulating proteins in unicellular eukaryotes that are easily amenable to genetic manipulations. Similar to mammalian cells undergoing apoptosis, yeast cells expressing Bax exhibit mitochondrial localization of the latter, cytochrome *c* release to the cytosol [19,20], and apoptotic phenotype [21] that are prevented by co-expression of anti-apoptotic protein Bcl-x_L [1,2,22]. Yet, mutants of Bcl-x_L, which retain the ability to bind Bax but which are deficient in anti-apoptotic activity in mammalian cells, are similarly unable to suppress Bax-induced effects in yeast [2]. These observations indicate that in yeast cells Bax may act directly upon conserved cellular components that correspond to their apoptotic targets in mammalian cells.

In this work, the effects of Bax expression on *Saccharomyces cerevisiae* cells were reexamined using mutants and physiological conditions that altered mitochondrial oxidative phosphorylation and mitochondrial biogenesis. In contrast to the results obtained by other investigators [17,22,23] our results demonstrate that neither fully active mitochondria, nor presence of AAC protein in mitochondrial membrane are required for Bax-induced growth defects in yeast cells. In addition, our results suggest that in *S. cerevisiae* Bax may intrude upon one essential cellular pathway involving targeting and import of proteins to the mitochondria.

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Abbreviations: AAC, ADP/ATP carrier; GFP, green fluorescent protein; SM, synthetic liquid medium; ANT, bovine adenine nucleotide translocator; DAPI, 4′,6-diamidino-2-phenyl-indole; PTP, permeability transition pore; VDAC, voltage-dependent anion channel; MTP1, mitochondrial telomere binding protein

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2. Materials and methods

2.1. Yeast strains, plasmids and growth conditions

The wild type strain W303-1B (*MAT*α, *ade2, leu2, his3, trp1, ura3, can1*) provided by B.L. Trumpower (Dartmouth Medical School, Hanover, NH, USA) was used for preparation of the mutants listed below; the *AAC2* disrupted strain JLY-73 (*MAT*α, *ade2, leu2, his3, trp1, ura3, can1, aac2::HIS3*), the triple *aac* mutant JL-1-3 (*MAT*α, *ade2, leu2, his3, trp1, ura3, can1, aac1::LEU2, aac2::HIS3, aac3::URA3*), and the mitochondrial respiratory deficient (ρ^0) mutant prepared by ethidium bromide mutagenesis.

The cDNA for mouse *bax* gene was cloned in YEp51 [19] and YpN2 [24] for Gal-promoter regulated expression of Bax in yeast. The shuttle vector pRS314 containing the bovine AAC gene, in which the region encoding N-terminal 13 amino acids of the bovine gene was substituted for the corresponding region of yeast *AAC2* gene [25], was used to express the hybrid yeast bovine protein in *AAC2* deleted (JLY-73) strain.

The plasmid pGFP-C-FUS-AAC1 containing the whole AAC1 open reading frame (lacking stop codon) fused with green fluorescent protein (GFP) was prepared by ligation of PCR product amplified with 5′-CGGGATCCATGTCTCACACAGAAACA-3′ and 5′-CGGAATTCCTTGAATTTTTTGCC-3′ primers, into *Bam*HI, *Eco*RI-digested pGFP-C-FUS vector ([26], provided by J.H. Hegemann, Justus-Liebig University, Giessen, Germany). The construction of pGFP-C-FUS-mitochondrial telomere binding protein (MTP1) plasmid containing the MTP1 reading frame is described in [27].

Yeast cells transformed with control (YEp51, YpN2) and Bax containing (YEp-Bax, YpN2-Bax) vectors were inoculated into synthetic liquid medium (SM, 0.67% yeast nitrogen base, Difco) supplemented with indicated carbon source and nutritional requirements, and grown until exponential growth phase. To induce Bax expression, the cells were harvested, washed twice with sterile water, diluted to 10^6 cells/ml into fresh minimal medium containing 2% galactose, and grown either aerobically at 30°C, or anaerobically as described before [24]. The plating efficiency (clonogenicity) of Bax-expressing and control-plasmid containing cells was estimated by aliquots taken out at different times after induction and plating 500 cells on solid 2% agar containing minimal (SM) or complex (YP) medium containing 1% yeast extract, 2% Bacto peptone (Difco), and supplemented with 2% glucose. The number of colonies was scored after 2–3 days of growth at 30°C.

2.2. Mitochondrial $\Delta\Psi$ assay, oxygen consumption, and GFP fluorescence

Yeast spheroplasts, prepared as in [28], were resuspended at concentration 1 mg protein/ml in a medium containing 1 M sorbitol, 0.5 mM EGTA, 2 mM $MgSO_4$, 1.7 mM NaCl, 10 mM potassium phosphate, 0.1% bovine serum albumin, pH 6.8 and permeabilized with 20 μg/ml nystatin (Sigma) for 10 min at 28°C as described in [29]. The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated in the permeabilized spheroplasts with rhodamine 123 (Molecular Probes) using a Shimadzu RF-5301PC spectrofluorimeter at 507 and 529 nm excitation and emission wavelengths, respectively. The oxygen consumption by permeabilized spheroplasts was measured using a Clark oxygen electrode in the same medium as described above.

Cells transformed with the GFP fusion containing plasmids were loaded with 4′,6-diamidino-2-phenyl-indole (DAPI, Molecular Probes) and observed at the indicated times using a Olympus BX-50 fluorescence microscope equipped with filters recommended by the manufacturer.

2.3. DNA manipulations and analysis

DNA isolations, labeling, cloning, sequencing, Northern blotting, and hybridization were performed essentially as described in [30]. The lithium acetate procedure was used for yeast transformations [31]. The enzymes for DNA manipulations, DNA labeling, and radiochemicals were purchased from Amersham Pharmacia Biotech.

3. Results

3.1. Bax induced inhibition of growth and clonogenicity of yeast cells

We focused on studying the effects of Bax in mitochondrial mutants of *S. cerevisiae* since previous studies have demonstrated an essential role of these organelles in mammalian apoptosis [5–9]. For this purpose yeast expression vectors containing Bax gene under Gal promoters were transformed into the wild type strain, as well as into the mutants strains with

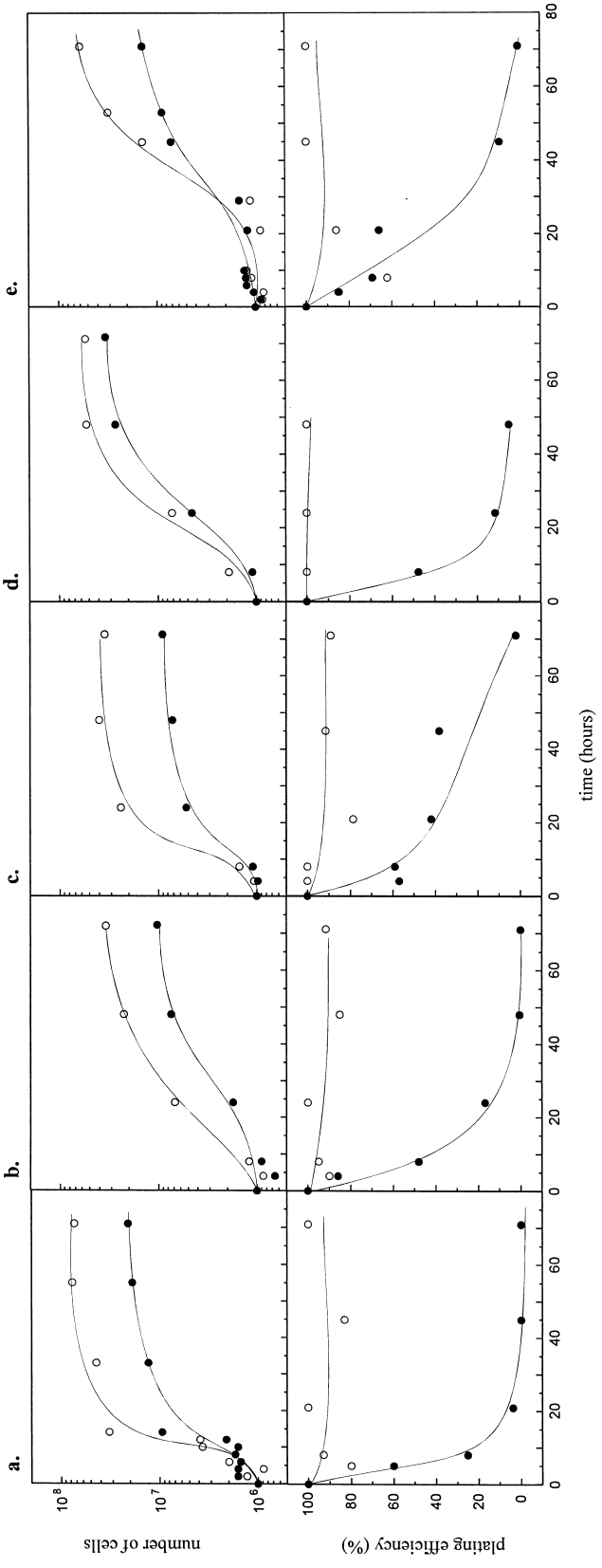

Fig. 1. Bax-induced inhibition of growth (upper panel) and plating efficiency (lower panel) of *S. cerevisiae* cells. (a) The wild type, (b) a respiratory deficient (ρ^0) mutant, (c) the *AAC2* deletion mutant (JLY-73), (d) the triple *aac* deleted strain (JL-1-3), and (e) the JLY-73 mutant expressing the hybrid yeast′bovine *AAC-ANT* gene were transformed with the control (open circles) and Bax containing (full circles) vectors and grown into 2% galactose containing SM medium as described in Section 2. The plating efficiency (lower panel) was evaluated at different times after Bax induction. Aliquots of the cultures were taken out, washed with sterile water and plated on a minimal 2% glucose containing solid medium. The number of colonies was scored after 2–3 days of growth at 30°C.
←

defects in mitochondrial oxidative phosphorylation. The latter included a respiratory deficient (ρ^0) mutant, two nuclear mutants in which either the major *AAC2* gene, or all three (*AAC1, AAC2, AAC3*) AAC encoding genes were deleted, and the AAC-less strain expressing a recombinant bovine *ANT2* (adenine nucleotide translocator) gene [25]. As shown in Fig. 1, the induction of Bax expression, by shifting the carbon source in the medium from glucose to galactose, resulted in unequal inhibition of growth yields and unequal increase of duplication time in wild type and mutant strains. Bax expressing wild type and mutant cells were not capable of recovering growth when plated on glucose containing SM. Inhibition of growth and plating efficiency were also observed in the mutant expressing recombinant bovine *ANT* gene (Fig. 1), as well as in the wild type strain grown under anaerobic conditions (not shown).

As the growth rate and the extent of Bax induced growth inhibition in different mutants varied, we evaluated the cytotoxic effect of Bax in relation to the number of cell divisions (Fig. 2). This demonstrates that in all tested strains 2–3 cell divisions in the presence of Bax are sufficient to achieve 90% decrease of plating efficiency at conditions requiring the plasmid maintenance (SM-2% glucose).

We concluded that mouse Bax gene inhibited the growth and plating efficiency of wild type and mutant yeast strains independently of the presence, or the absence of functional oxidative phosphorylation and/or AAC proteins in their mi-

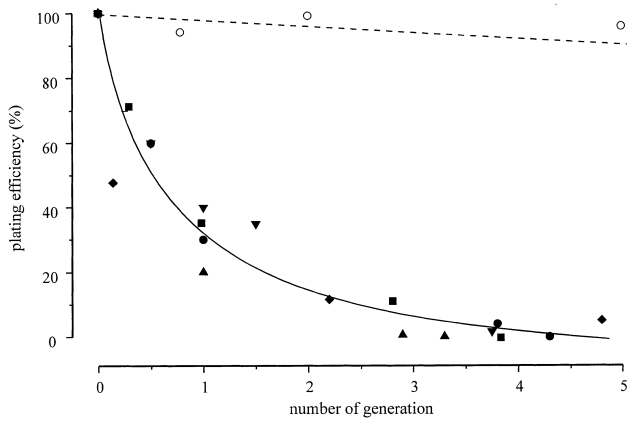

Fig. 2. Relationship between plating efficiency and number of cell divisions in the presence of Bax. Wild type transformed with the control plasmid (-○-). The wild type (-●-), the respiratory deficient (ρ^0) mutant (-▲-), the *AAC2* deletion mutant (-▼-), the triple *aac* deleted strain (-◆-), and the strain expressing the hybrid *AAC-ANT* gene (-■-) transformed with Bax containing plasmid.

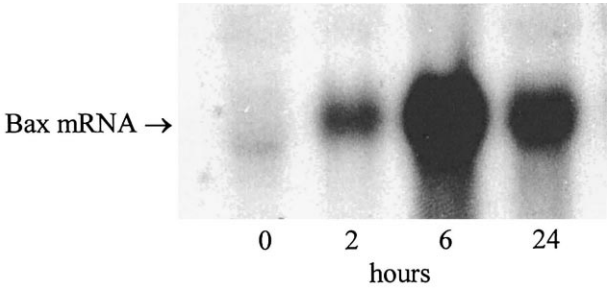

Fig. 3. Northern blot analysis of the Bax mRNA levels. The total RNA from the wild type strain transformed with YEp-Bax was isolated at the indicated time after galactose induction. The RNA (10 μg) was denatured, electrophoresed, blotted and probed as described [24] with a ^{32}P-labelled fragment from the murine Bax gene.

tochondria. As both cell division and Bax expression in galactose containing media depend on Gal promoter, it is conceivable that the different effect of Bax on the growth of mutant cells is due to the different strength of the promoter under different physiological conditions.

3.2. *The expression of Bax in S. cerevisiae inhibits mitochondrial respiration but does not dissipate the mitochondrial transmembrane potential ($\Delta\Psi$)*

Northern blot analysis indicates that the wild type cells transformed with YEp-Bax express a high level of Bax mRNA in a short time after galactose induction (Fig. 3). Therefore, to estimate the early effects of Bax on the mitochondrial functions, the cells were harvested 6 h after Bax induction, converted to spheroplasts, and permeabilized with nystatin [29]. The latter treatment enabled the measurements of mitochondrial membrane potential and respiration without purification procedures that could eventually eliminate a part of the organelle's population affected by Bax. As shown in Fig. 4a, the mitochondria in Bax expressing cells were capable of developing mitochondrial transmembrane potential ($\Delta\Psi$) with a magnitude similar to that observed in the control cells. In contrast to the latter, however, the addition of ADP to mitochondria in Bax expressing cells does not produce a decrease of $\Delta\Psi$. The drop of $\Delta\Psi$ in the control cells probably reflects its consumption during ADP transport and phosphorylation. Additions of either oligomycin, or atractylate to the control protoplasts (not shown) can mimic the effect of Bax expression indicating that Bax affects the oxidative phosphorylation of yeast mitochondria. This is further substantiated by assessment of the respiration rates of nystatin treated spheroplasts (Fig. 4b). The oxygen consumption of spheroplasts prepared from Bax-expressing cells proceeds with a maximal rate in the absence of ADP and uncoupler. It represents only 50% of the maximal respiration rate of cells transformed with the control plasmid. The diminished respiration rate is in agreement with the reduced content of cytochrome *c* oxidase observed in Bax expressing cells [20]. The features of mitochondria in Bax expressing yeast cells thus confirm that Bax protein interacts with mitochondrial membranes and affects the mitochondrial function.

3.3. *The estimation of possible Bax effect on the mitochondrial protein import*

Taking into account that mitochondrial biogenesis in yeast cells is an essential process, we have investigated the effect of

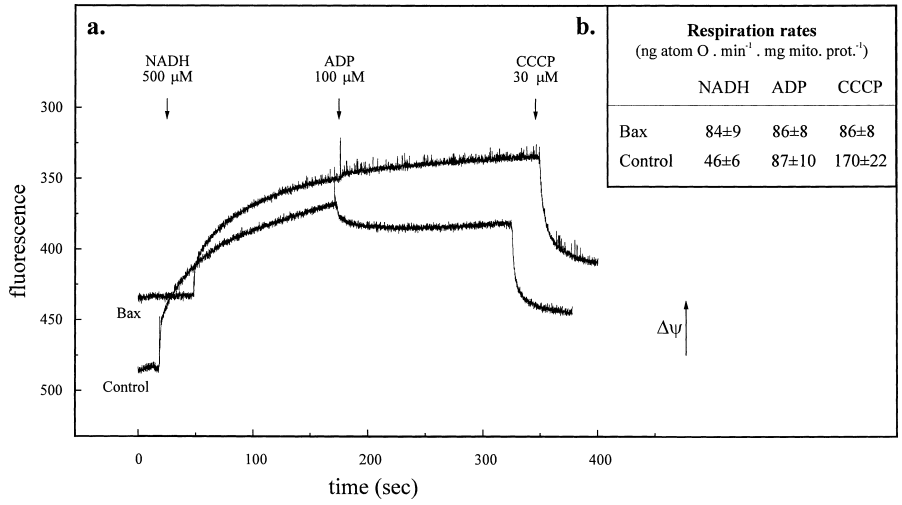

Fig. 4. The expression of Bax affects mitochondrial functions in yeast cells. The wild type cells transformed with either YEp-Bax, or YEp51 plasmids were harvested 6 h after galactose induction, converted to spheroplasts by enzymatic digestion as described in [28], and treated with nystatin to permeabilize the cell plasma membrane. a: The changes of mitochondrial $\Delta\Psi$ were assessed by following rhodamine 123 fluorescence as described in Section 2. b: The respiration rates of permeabilized spheroplast were measured by a Clark oxygen electrode.

Bax on the import of protein to the mitochondria. For this purpose we have used GFP fusions with two mitochondrial proteins – the MTP1 that contains a cleavable N-terminal targeting sequence [27], and the AAC1 that belongs to the mitochondrial carrier family of proteins possessing internal targeting sequences and taken up by mitochondria by a different import pathway [32,33]. To estimate the effect of Bax on the import of these two kinds of fusion proteins, the cells were co-transformed with Bax carrying plasmids, cultured on SD-Gal medium to induce Bax expression, and analyzed for fluorescence at different time intervals. Both MTP1–GFP and AAC1–GFP fusion proteins localize to the mitochondria as demonstrated by DAPI staining of mtDNA (Fig. 5B,C,B′,C′). The induction of Bax resulted in the appearance of green–yellow fluorescent cells that are also homogeneously stained by DAPI (Fig. 5E–I, E′–I′). As such cells were rarely seen in the control vector (YEp51) containing culture (Fig. 5K′,L′) and were also stained by methylene blue (data not shown), they probably represent the non-viable cell population as a consequence of Bax action. The number of these cells increases with the time after Bax induction, but it remains roughly equal in both MTP1–GFP and AAC1–GFP containing cells.

In addition, the inspection of Bax effect on GFP fluorescence provided dissimilar pictures originating from both MTP1–GFP and AAC1–GFP fusions. Almost all viable cells co-expressing Bax and MTP1–GFP for 24 and 48 h (Fig. 5E,H) exhibited an intensive mitochondrial fluorescence. At the same time, the number of cells with visible mitochondrial fluorescence, co-expressing Bax and AAC1–GFP, was markedly diminished (Fig. 5E′,H′). In the control cells that do not express Bax, the fluorescence yielded by AAC1–GFP remained basically unchanged (Fig. 5K′). These observations suggest that either the AAC1–GFP fusion is faster and specifically degraded by the cellular proteases in Bax expressing cells, or the import of mitochondrial carrier proteins is preferentially affected by Bax as compared to the import of proteins containing an N-terminal targeting sequence.

4. Discussion

Although no obvious homologues of any important regulator of metazoan apoptosis in the *S. cerevisiae* genome were detected, the expression of the latter two classes of proteins induces growth defects and cellular phenotypes characteristic for metazoan apoptotic cells [19–23]. It was suggested that the cytotoxic effects of Bax in yeast cells are relevant to its mechanism of action in mammalian cells and that it most probably involves mitochondria as a target for proapoptotic protein action. The results presented in this study confirm and extend the previous published observations that expression of Bax in yeast inhibited the cell divisions and affected the mitochondrial functions in this organism [20–23]. In addition, we showed that the effect of Bax on *S. cerevisiae* does not require mitochondrial functions associated with the oxidative phosphorylation. This is demonstrated here by the experiments showing that (i) the wild type respiratory competent cells, (ii) the respiratory deficient (ρ^0) mutant lacking mitochondrial DNA, (iii) AAC-less mutants lacking AAC proteins in inner mitochondrial membrane, and (iv) anaerobically-grown wild type cells, were all affected by Bax in a similar manner. In addition, our results demonstrate that the interaction between mammalian AAC and Bax protein, previously demonstrated by the two-hybrid assay and co-immunoprecipitation [17], has not substantially changed the cytotoxic effect of Bax in yeast cells. These observations contradict the findings published by others that fully active mitochondria [22,23], or mitochondrial AAC [17] are essential for Bax induced cytotoxicity in yeast. The discrepancies between the latter works and these presented here are probably due to the fact, that the enzymes involved in galactose metabolism are repressed in cells with impaired mitochondrial functions and consequently Gal promoter does not function properly in these cells. Recent work [34] using a tetracycline inducible promoter to express Bax in the AAC-less mutant has indeed shown that the absence of AAC did not modify Bax-induced lethality.

The effect of Bax on both the mitochondrial respiration and

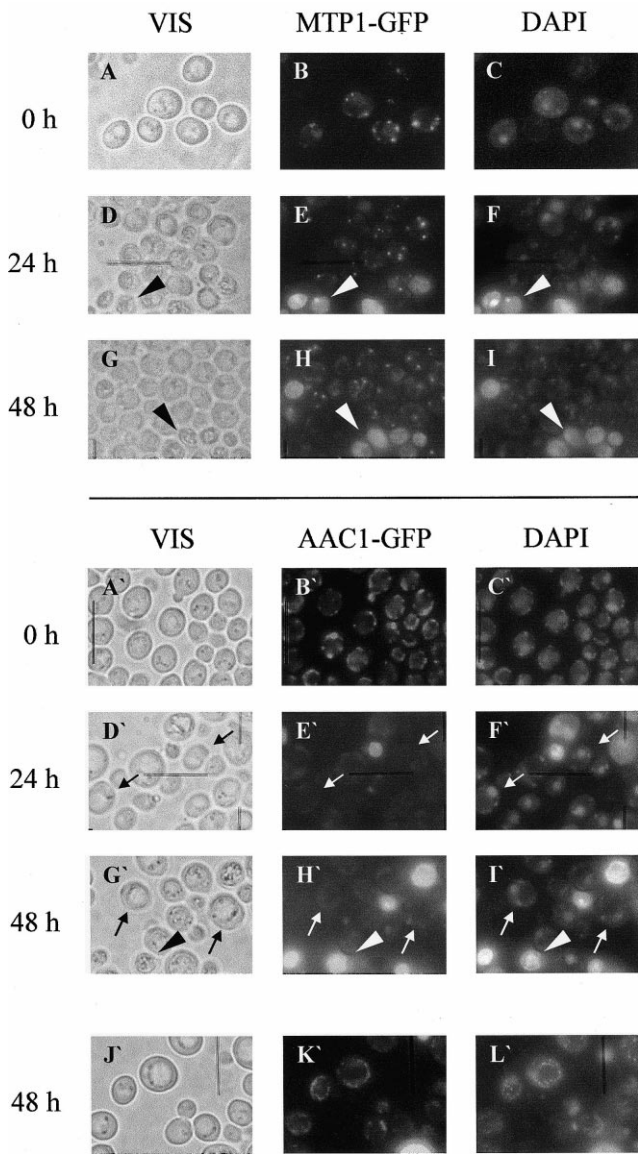

Fig. 5. The expression of Bax has a different effect on the mitochondrial fluorescence yielded by MTP1–GFP and AAC1–GFP fusion proteins. The wild type (W303-1B) yeast cells co-transformed with YEp-Bax plus pGFP-C-FUS-MTP1 (A–I), with YEp-Bax plus pGFP-C-FUS-AAC1 (A′–I′), or with YEp51 plus pGFP-C-FUS-AAC1 (J′–L′) were induced to express Bax by resuspending the cells in SM-galactose medium (0 h). At specified time intervals (24 h and 48 h), samples were withdrawn, stained with DAPI, and analyzed directly by fluorescence microscopy using appropriate filters for visualization of the green (GFP) and blue (DAPI) light. Both MTB1–GFP and AAC1–GFP localize to the mitochondria (also stained by DAPI). The number of non-viable cells (pointed by arrowheads) exhibiting dispersed yellow–green (E, H, H′) and blue (F, I, I′) fluorescence was equal in both types of fusion containing cells and it is increasing with the time after Bax induction. The cells pointed by the arrows are examples of viable cells that lack mitochondrial fluorescence. An increasing number of these cells is observed among the Bax expressing, AAC1–GFP fusion containing cells (E′, H′), while almost all viable, MTP1–GFP containing cells (E, H) clearly exhibit mitochondrial fluorescence after 24 and 48 h of Bax expression. The same cells were examined under visible light, with DAPI, and a green fluorescence filter.

the properties of mitochondrial transmembrane potential (Fig. 4) indicates that Bax interacts with the yeast mitochondrial proteins and/or lipids. The pleiotropic effect of this interaction also include cytochrome *c* release from the intermembrane space of yeast [20] and mammalian mitochondria [7,8], indicating that outer mitochondrial membrane belongs among the Bax targets in both type of cells. The question is, however, how could the impaired mitochondrial functions account for the cytotoxic effect of Bax on yeast cells?

S. cerevisiae are facultative anaerobes and can easily survive mitochondrial defects as these described above. In yeast, mitochondria can affect the cell division by one of the following two pathways; first, by simultaneous inhibition of both ADP/ATP transport and respiration that leads to depletion of the intra-mitochondrial ATP pool [35] and second, by mutations of mitochondrial proteins participating in the import of proteins to the mitochondria (for review see [36]). The intra-mitochondrial ATP pool and protein import machinery are both required for mitochondrial biogenesis and they are the only so far known essential mitochondrial constituents. Therefore, they might represent attractive targets for the execution of mitochondrial control in cell death. Our experiments in which we followed the mitochondrial fluorescence yielded from two mitochondrial proteins fused to GFP provide support for Bax action on the mitochondrial protein import process. The faster extinguishing of mitochondrial AAC1–GFP fluorescence in larger number of Bax expressing cells, as compared to the extinction of MTP1–GFP, suggests that Bax preferentially inhibits the import of carrier proteins to yeast mitochondria. Although, other explanations might be also offered (e.g. different susceptibility of the recombinant proteins to cellular proteases), our presumption that Bax may affect preferentially the import of carrier proteins can find support in other experimental results. It is known in yeast and mammalian cells that Bax increases the permeability of outer mitochondrial membrane and release proteins that are located at the intermembrane space of mitochondria [7,8,20,37]. At the same time in vitro experiments with isolated yeast mitochondria demonstrated that intact outer membrane is essential for the import of carrier proteins, but not for the import of N-terminal targeting sequence containing proteins [38]. Thus Bax induced permeabilization of outer mitochondrial membrane in vivo might have a similar effect on the import of carrier proteins to yeast mitochondria. One can hypothesize further that the inhibition of the import can be executed by one of the two alternative ways. First, by Bax induced permeability of outer mitochondrial membrane and release of essential soluble components (TIM9, TIM10, TIM12, TIM8) of import machinery [32,33,36,38]. The second possibility might be that Bax simply compete and preferentially occupy the protein import machinery that is specific for mitochondrial carrier proteins. The work to test these possibilities is now in progress in our laboratory. Whatever the mechanism underlying Bax effect on the mitochondrial carrier proteins import, it provides a link between the two observed Bax induced phenomena – the mitochondrial defects and the growth inhibition of yeast cells.

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