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Expression of Bax in yeast affects not only the mitochondria but also vacuolar integrity and intracellular protein traffic

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Abstract Bax-induced lethality in yeast is accompanied by morphological changes in mitochondria, giving rise to a reduced number of swollen tubules. Although these changes are completely abolished upon coexpression of the Bax inhibitor, Bcl-2, coexpression of Bax with Bax inhibiting-glutathione *S*-transferase (BI-GST) leads to aggregation, but not fusion of the mitochondria. In addition, Bax affects the integrity of yeast vacuoles, resulting in the disintegration and eventual loss of the organelles, and the disruption of intracellular protein traffic. While Bcl-2 coexpression only partially corrects this phenotype, coexpression of BI-GST fully restores the organelles, indicating a different mode of protection exerted by Bcl-2 and BI-GST.

Keywords: Programmed cell death; Yeast; Bax; Glutathione *S*-transferase; Mitochondria; Vacuole

1. Introduction

The Bcl-2 family of proteins is at the core of the metazoan cell death machinery. It includes pro-survival and pro-apoptotic members, several of which appear to have an impact on survival pathways in heterologous systems that do not contain endogenous Bcl-2 proteins. Plants expressing Bcl-2 and Bcl-X_L, or CED-9 display resistance to several necrotrophic fungal pathogens and to a necrogenic virus [1–3]. In yeast, Bcl-2 and CED-9 inhibit oxidative stress-induced programmed cell death (PCD) [4]. Conversely, Bax-expression in plants causes localized tissue collapse in a manner resembling the hypersensitive response, a PCD response of plants in defense against pathogens [5]. Moreover, human or plant genes isolated from yeast genetic screens for Bax or Bak suppression have been associated with pro-survival effects when expressed in their native or a heterologous context [6–9].

Bax, a mammalian proapoptotic member of this family, is thought to alter organelle function by localizing to the outer mitochondrial membrane and forming an ion channel [10]. Yeast cell death caused by Bax, and its close relative Bak, appears relevant to its function in a physiological setting [11]. In a screen to identify plant inhibitors of Bax lethality in yeast, we identified a glutathione *S*-transferase (GST), named Bax Inhibiting-GST (BI-GST). In yeast, coexpression of BI-GST with Bax restored glutathione to wild-type levels and preserved mitochondrial function, while BI-GST expression protected cells from prooxidant insults [12,13]. This study investigates the effects of Bax expression on yeast organelles. Changes in mitochondrial morphology and function, together with disruptive effects on the vacuole and on protein trafficking, are reported. The suppression of Bax lethality by Bcl-2 and BI-GST appears to be achieved in a different mode in each case. Bcl-2 mainly protects the mitochondria, whereas BI-GST maintains the integrity of the vacuoles.

2. Materials and methods

2.1. Flow-cytometric studies

EGY48 cells harboring the LexA-Bax construct (pGILDA/Bax) in conjunction with pJG4-6/BI-GST and cells harboring only the BI-GST construct were grown, stained, and processed as previously described [12]. Dihydroethidium (HE; D-1168, Molecular Probes) at 4 μM was used as an indicator of endogenous reactive oxygen species (ROS).

2.2. Fluorescent microscopy of yeast organelles

Mitotracker Red CMxROS (M-7512, Molecular Probes) was used to stain mitochondria at a final concentration of 2 μM for 20 min. Vacuolar membranes were stained with 4 μM MDY-64 (Y-7536, Molecular Probes) for 5 min. The vacuolar lumen was stained with 50 μM CMAC (Y-7531, Molecular Probes) for 20 min. Cells were washed twice, resuspended in prewarmed PBS, applied on microscope slides, and observed at 1000× magnification. To visualize the mitochondrial matrix, plasmid pVT100U-mitGFP (which expresses GFP fused to a mitochondrial matrix targeting sequence [14]) was introduced to yeast cells. To monitor aspects of the vacuolar transport pathway, plasmid GFP-alkaline phosphatase (ALP) (expressing a GFP fusion of the vacuolar membrane protein ALP) was used [15].

2.3. Expression of BI-GST and Bax as GFP fusions in yeast

The ORF of GFP was PCR-amplified using primers that introduce restriction sites for *Eco*RI and *Sal*I at the 5′ of the cDNA. The PCR product was cloned into the pCRT7/CT TOPO vector (Invitrogen). The ORF of BI-GST from the pBad/BI-GST vector [12] was inserted

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Abbreviations: PCD, programmed cell death; ROS, reactive oxygen species; GST, glutathione *S*-transferase; BI-GST, Bax inhibiting-GST; HE, dihydroethidium; ALP, alkaline phosphatase; EA, ethacrynic acid; BSO, buthionine sulfoximine; $\Delta\Psi_m$, mitochondrial membrane potential

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in the pCRT7/CT-GFP construct using the *Eco*RI–*Sal*I restriction sites, fusing it in frame to the N-terminus of GFP. The BI-GST/GFP insert was subcloned into the *Eco*RI/*Xho*I sites of the yeast expression plasmid pJG4-6. The ORF of Bax from the pGILDA/Bax vector was subcloned in frame to the 3′ of the EYFP cDNA, which was previously inserted in the pJG4-6 yeast vector. Induction of EYFP-Bax caused yeast lethality, confirming functionality of the Bax fusion.

3. Results and discussion

3.1. Coexpression of BI-GST restores viability and removes ROS

To assess the effect of BI-GST on the viability of yeast cells expressing Bax, cells growing in liquid cultures were stained with trypan blue and enumerated by microscopy. After expression of Bax for 24 h, ~25% of cells were dead. The remaining cells were in a state of stasis, at the point of the cell cycle in which LexA-Bax induction first occurred, and were unable to divide [12]. In cells coexpressing BI-GST, cell-death was reduced to ~10% after 24 h (Fig. 1A).

Bax-expression is known to cause a transient increase in ROS, followed by a drop to slightly lower than wild-type levels [12]. To assess the effect of BI-GST in intracellular ROS, yeast cells were stained with HE, a compound used to probe oxidation caused by the respiratory burst, and the percentage of positively staining cells was determined by FACS analysis. When wild-type cells were examined, 21% stained positive, while addition of FCCP, an uncoupler that dissipates the proton gradient, reduced the number of positive cells to 16%, indicating that the fluorescence was mostly due to respiration (Fig. 1B). Expression of Bax increased the number of positive cells to 33%, while expression of BI-GST reduced the number of positive staining cells both in the absence and in the presence of Bax (16% and 27%, respectively).

3.2. Suppression of Bax lethality does not depend on YAP1, ethacrynic acid (EA), or buthionine sulfoximine (BSO)

The prooxidant protective capacity of BI-GST depends on the presence of YAP1, a yeast transcriptional regulator of the oxidative response [13]. However, deletion of *YAP1* does not abolish the capacity of BI-GST to suppress Bax lethality, indicating the presence of distinct pathways for the protection against prooxidants and Bax expression (Fig. 1C). To examine the effect of the glutathione detoxification system on the ability of BI-GST to suppress Bax, cells were plated on media supplemented with increasing concentrations of BSO, a compound which reduces intracellular levels of GSH, and EA, a reversible inhibitor of GSTs. Neither compound had any effect on the Bax-suppressing capacity of BI-GST (data not shown).

3.3. Mitochondrial alterations

Bax expression in mammalian and yeast cells is characterized by the disruption of mitochondrial function and the dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) [12,16]. In yeast, at the early stages of Bax-expression, $\Delta\Psi_m$ becomes hyperpolarized and progressively collapses [12,17]. $\Delta\Psi_m$ and mitochondrial morphology were assessed using the Mitotracker Red fluorophore. In cells expressing Bax, the dye was found to concentrate in fewer mitochondria, which were significantly larger in size compared to wild-type cells (Fig. 2). Coexpression of Bcl-2 restored mitochondria to wild-type appearance. While expression of BI-GST alone did not have any

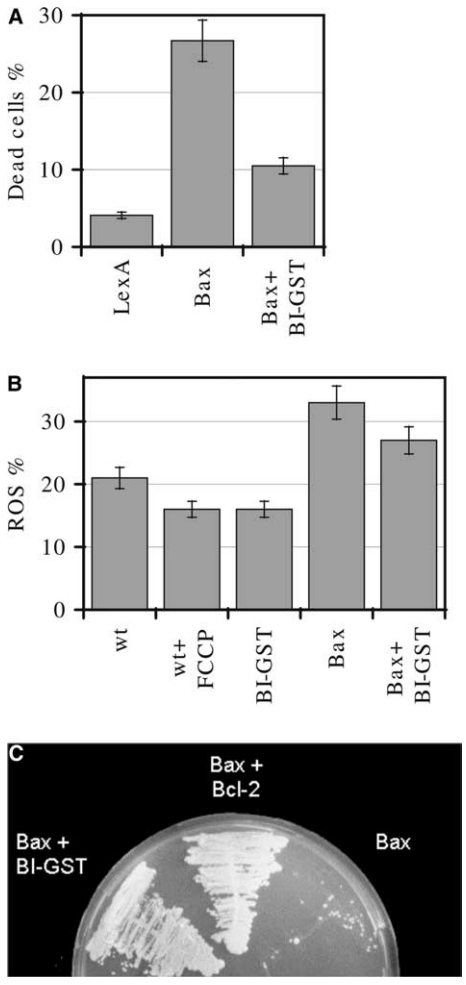

Fig. 1. Changes in viability and intracellular ROS. (A) EGY48 cells expressing LexA-Bax in the presence and absence of BI-GST were grown in liquid gal-raff/CM medium. Cells carrying empty pGILDA plasmid, expressing the LexA protein, were used as control. Aliquots of cells were stained with trypan blue, a dye used as an indicator of viability, and the percentage of dead cells was enumerated. (B) Cells were grown as above, stained with the oxidation sensitive probe HE, and subjected to flow cytometry to determine the percentage of positive cells. (C) Δ*YAP1* cells expressing LexA-Bax with BI-GST or Bcl-2 were plated on gal-raff/CM-his, trp media. The absence of YAP1 had no effect on the protective capacity of BI-GST or Bcl-2.

effect on mitochondrial morphology, coexpression of Bax and BI-GST resulted in clustered mitochondria. These mitochondrial alterations were studied further using a plasmid expressing GFP fused to a mitochondrial matrix targeting sequence [14]. Wild-type cells showed bright peripheral GFP fluorescence of several thin tubules representing the mitochondria (Fig. 2). Expression of Bax, as in the case of Mitotracker staining, caused a dramatic change in the pattern of fluorescence characterized by a decreased number of swollen mitochondria. Coexpression of Bcl-2, again, caused complete reversal of the mitochondrial morphology to wild type appearance. In contrast, coexpression of BI-GST caused an aggregation of brightly fluorescing and large in diameter tubules, which in most cases maintained their integrity and did not fuse.

Changes in mitochondrial morphology have been reported in numerous systems, frequently resulting from dysfunctions in

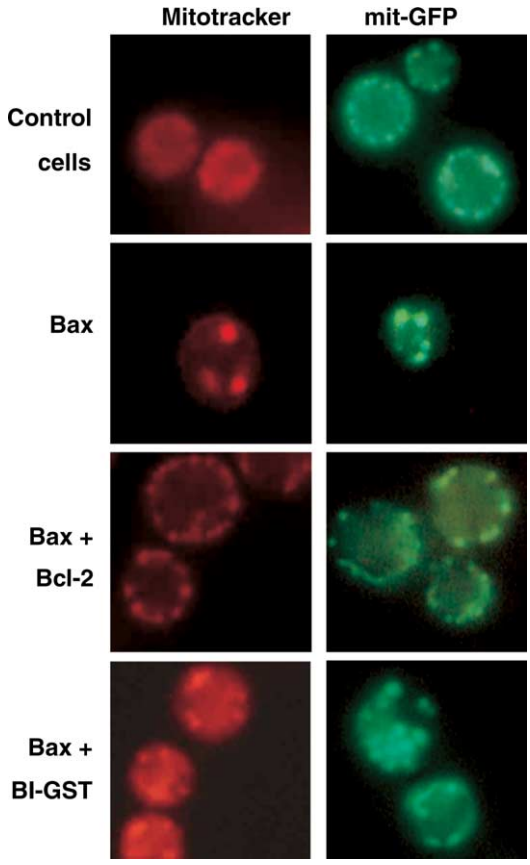

Fig. 2. Mitochondrial alterations caused by Bax. (Left) EGY48 cells expressing LexA-Bax alone or together with Bcl-2 or BI-GST were stained with Mitotracker, a dye that localizes to the mitochondria as a consequence of $\Delta\Psi_m$. (Right) Plasmid pVT100U-mitGFP, which expresses GFP fused to a mitochondrial matrix targeting sequence, was introduced to the above-mentioned cells to monitor mitochondrial morphological alterations.

the energy supply machinery [18]. Alterations in mitochondrial morphology caused by Bax have also been identified in mammalian cells. Bax has been shown to be present in mitochondrial constriction sites, separating mitochondria into several shorter units and promoting apoptosis [19].

3.4. Vacuolar alterations

The recent identification of a Bax-tolerant yeast mutant in the ADP-ribosylation factor-like protein, ARL1, suggested a potential involvement of the vacuole [20]. To assess the effect of Bax and BI-GST on vacuolar morphology, we stained cells with the yeast vacuole membrane fluorophore marker MDY-64, the blue fluorescent dye CMAC (which accumulates in the lumen of the vacuole), and the GFP-tagged ALP (which labels specifically the vacuolar membrane and can be used to monitor aspects of protein traffic to the vacuole).

Wild-type EGY48 cells, stained with MDY-64 and CMAC, show one or two well-structured vacuoles (Fig. 3). In a large number of CMAC stained cells, small endosomal vesicles in the process of fusion can clearly be seen. Intense ALP-GFP fluorescence is evident on the vacuolar membranes. Bax-expressing cells exhibit dramatic alterations in vacuolar morphology. At the early stages of protein expression, vacuoles become intensely fluorescing non-canonical structures with

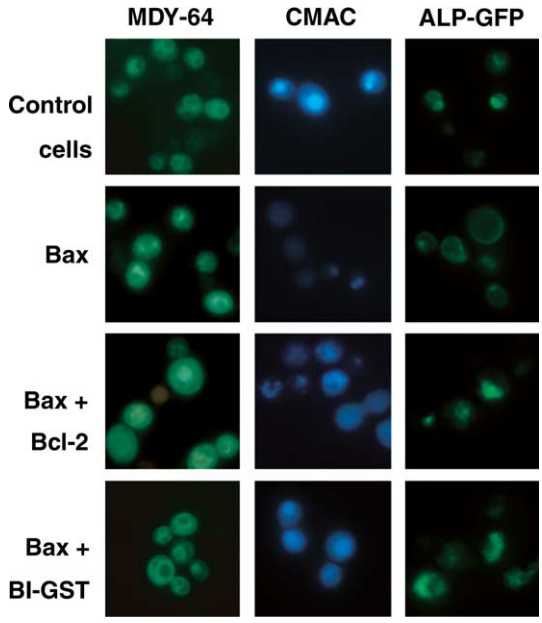

Fig. 3. Vacuolar morphology of cells expressing Bax. Effects of BI-GST and Bcl-2. EGY48 cells expressing LexA-Bax alone or together with Bcl-2 or BI-GST were stained with MDY-64 and CMAC to visualize the vacuolar membrane and lumen, respectively. Protein transport to the vacuole was monitored by transforming cells with a plasmid expressing an ALP-GFP fusion.

foamy appearance (Fig. 3). At later stages, most cells lose MDY-64 fluorescence and only few show faintly stained vacuoles. CMAC staining revealed a similar picture, consisting of highly fluorescing cells with fragmented vacuoles and a progressively increasing number of cells that do not fluoresce. CMAC does not stain dead cells. However, the proportion of dead cells (~25%, Fig. 1A) is substantially less than that of non-fluorescing cells (~65%), indicating the presence of a population of viable cells that lack functional vacuoles. After 24 h of Bax expression, ALP-GFP fluorescence is lost from vacuolar membranes giving rise to diffused cytoplasmic staining. A population of cells fluoresces on the cytoplasmic membrane, indicating missorting of the ALP protein. When Bcl-2 is coexpressed with Bax, cells exhibit a similar but not identical morphology to those expressing Bax alone, with a larger proportion of cells maintaining CMAC and ALP-GFP fluorescence. Unlike Bcl-2 coexpressing cells, the cells that coexpress BI-GST have their vacuolar structure preserved, most of them showing one or two large vacuoles. Staining with CMAC revealed an intermediate intensity and regular vacuolar morphology, while intense fluorescence of the ALP-GFP was seen in vesicles proximal to the main vacuole.

To examine the site of action of Bax and BI-GST in yeast cells, we expressed a C-terminal GFP fusion of BI-GST and an N-terminal EYFP fusion of Bax. At the initial stages of protein induction, BI-GST-GFP exhibited diffused cytoplasmic fluorescence. At later stages, this concentrated in small vesicles in the proximity of the main vacuole, but it did not fuse to the latter (Fig. 4A). Fluorescence in structures that may represent mitochondria could also be seen. EYFP-Bax fluorescence was substantially weaker. It could be detected in mitochondrial tubules at the periphery of the cell and as a more diffused stain

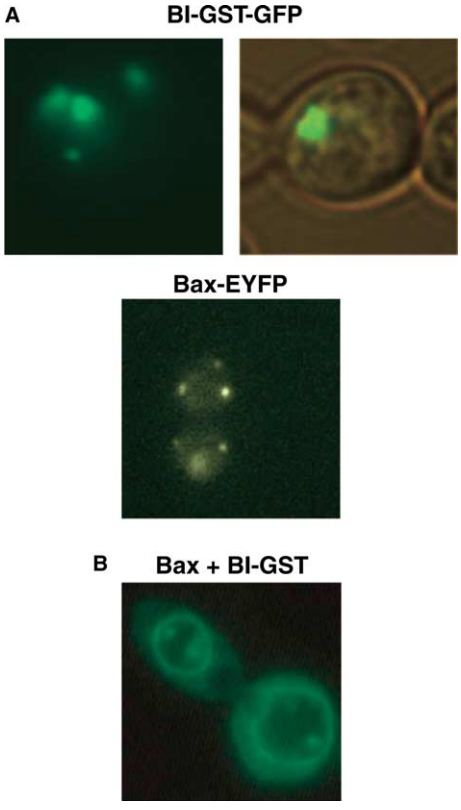

Fig. 4. (A) Intracellular localization of BI-GST and Bax. (Top) EGY48 cells expressing a BI-GST-GFP fusion were visualized by fluorescent microscopy (top left) and low intensity bright-field/fluorescent microscopy (top right). (Bottom) EGY48 cells expressing a Bax-EYFP fusion visualized by fluorescent microscopy. (B) Cells coexpressing Bax and BI-GST exhibit enhanced autophagy. MDY-64 staining of EGY48 cells coexpressing Bax and BI-GST reveals vacuolar membrane invaginations.

in the ER. The initial localization of BI-GST in the cytoplasm and its subsequent concentration in vesicles proximal to the vacuoles could justify its dominant protective effect on the organelles.

3.5. Autophagy

Cells coexpressing Bax with BI-GST appear to exhibit extensive autophagy. MDY-64 staining of the vacuolar membrane of cells coexpressing Bax and BI-GST reveals membrane invaginations, a characteristic of autophagic processes (Fig. 4B). Evidence for the link between autophagic processes and Bax expression in yeast has come from the recent discovery of Bax-resistant yeast mutants in which nutrient recycling processes associated with autophagy are compromised [21]. Autophagy is a unique, membrane-trafficking process that occurs in response to dramatic changes in nutrient availability in yeast cells, and is also known to occur in plant and animal cells [22]. In some mammalian systems, active autophagy appears to increase the tendency of cells to undergo PCD [23]. In yeast cells, rapid removal of damaged membranes and organelles by autophagy may be important for maintaining cellular viability. Being the final compartment in converging transport pathways, the vacuole receives endocytic traffic from the cell surface, biosynthetic traffic from the Golgi and material from the cytoplasm by autophagy [24].

The integrity of the lysosome, the mammalian counterpart of the yeast vacuole, has recently been implicated in the onset of the apoptotic process. Studies have shown that lysosomal rupture is an early event when apoptosis is initiated by lysosomal damaging agents, oxidative stress, oxidized lipids, serum withdrawal, Fas ligation and p53-induced apoptosis [23,25,26]. Overexpression of Bcl-2 can inhibit induction of lysosomal damage by oxidative stress [27]. In this report, Bax expression in yeast is shown to promote missorting of vacuolar proteins and disruption of the vacuole. These effects of Bax are likely to originate at the ER and the Golgi. BI-GST and Bcl-2 appear to exert their protective effect by a different mode, Bcl-2 predominantly on the mitochondria whereas BI-GST on the upstream events affecting vacuolar maintenance. Recent examination of mutant yeast strains, resistant to Bax, identified molecular events involved in Bax lethality that are distinct between mitochondria and the vacuoles [21].

4. Conclusion

Oxidative stress appears to be linked to the Bax phenotype. However, the effects of Bax in yeast cells are pleiotropic and involve at least two organelles and the delivery pathway. BI-GST was able to mediate removal of endogenous ROS both in the presence and absence of Bax. Unlike the protective effect that BI-GST exerts against prooxidants, which is YAP1-dependent, Bax suppression by BI-GST is YAP1-independent, suggesting an additional facet of BI-GST function which has not been elucidated. A connection between ROS production, mitochondrial localization, vacuolar function, and autophagy, with replicative senescence has been proposed in the past for yeast cells. Disruption of the autophagic pathway in plants is also linked to senescence [28]. Taken together, these results suggest the presence of conserved underlying pathways of cell death among eukaryotes.

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