Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in *Saccharomyces cerevisiae*

William Greenhalf, Christine Stephan, Bhabatosh Chaudhuri*

Department of Core Drug Discovery Technologies (CDDT), Ciba-Geigy AG, Basel, Switzerland

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Abstract In mammalian cells, the Bcl-2 and Bcl-x(L) proteins suppress programmed cell death whereas the topographically similar Bax protein accelerates the apoptotic process. Recently published data suggest that expression of the human *Bax-α* gene is lethal for the yeast *Saccharomyces cerevisiae* and that this toxicity can be overcome by co-expressing Bcl-2 or Bcl-x(L). Our findings corroborate these results. However, we find that although Bax induction invariably stops cell growth under all circumstances, it does not lead to death in 'petite' cells. Petites cannot respire because they lack functional mitochondria. It seems that in 'grande' cells, which do possess normal mitochondrial DNA, nutritional limitation is critical for increased mortality. Surprisingly, murine Bcl-2 lacking the membrane anchor of human Bcl-2 has no effect on grande cells, but can efficiently rescue petites in rich medium. It has been suggested that the C-terminal membrane anchor of human Bcl-2 may have a crucial role in rescuing apoptosis in mammalian cells. When murine Bcl-2 is fused to the membrane anchor of yeast mitochondrial Mas70 protein, the Bcl-2 variant mBcl-2–mma rescues not only petites but also grandes, just like human Bcl-x(L). The rescuing ability of Bcl-x(L), which contains its own membrane anchor, surpasses that of mBcl-2–mma. Our results indicate that the process involving Bax-induced growth inhibition followed by possible lethality, and the rescuing effect of Bcl-2 or Bcl-x(L) is linked to yeast mitochondrial function. We propose a model which is consistent with these observations.

Key words: Apoptosis; Bax; Bcl-2 family; Membrane-anchor; Mitochondrion; *Saccharomyces cerevisiae*

1. Introduction

Programmed cell death (PCD) in multicellular organisms is essential in development and can act as a final safety mechanism to eradicate 'sick' cells (i.e. cancer cells or infected cells) [1–4]. In mammals, the onset of PCD is characterized by a series of morphological changes in the affected cell. These changes are referred to as apoptosis and include membrane blebbing, autophagocytosis and selective DNA fragmentation at the junctions of nucleosomes [1].

*Corresponding author. Fax: (41) (61) 696 63 23.

Abbreviations: Bax, protein encoded by human *Bax-α* gene; Bcl-x(L), human Bcl-x(L) protein; Bi, yeast strain with integrated copy of *GAL10p–Bax* expression cassette; *CYP1p*, promoter from yeast cytosolic cyclophilin gene; *GAL10p*, promoter from yeast *GAL10* gene; Gal, galactose; *GAPDHp*, promoter from yeast glyceraldehyde-3-phosphate dehydrogenase gene; Glu, glucose; hBcl-2, human Bcl-2 protein; hma, human Bcl-2 membrane anchor; mBcl-2, murine Bcl-2 protein; mma, yeast Mas70 protein membrane anchor; PCR, polymerase chain reaction; PCD, programmed cell death.

The Bcl-2 protein counters a variety of stimuli which induce apoptosis [1,2]. Bcl-2 has turned out to be the founding member of a family of proteins. Some of the proteins of this family also contain a stretch of hydrophobic amino acids in their C-terminus that allows for post-translational insertion into intracellular membranes of the mitochondrion, the nucleus and the endoplasmic reticulum [5,6]. It has been shown that the C-terminal tail is important for Bcl-2's cell death blocking activity in mammalian cells [5–7].

The Bcl-2 family [4, 8–13] includes Bax, a homologous protein that dimerizes with Bcl-2 and when overproduced promotes apoptosis [4]. Bax also interacts with Bcl-x(L) which is known to suppress cell death [4,8].

Recently, it was reported that Bax induces growth arrest in the yeast *Saccharomyces cerevisiae* [14,15]. Quite surprisingly, this arrest is rescued by Bcl-2, Bcl-x(L) and Mcl-1 proteins, suggesting that important components of the apoptotic process may be conserved from a simple unicellular eukaryote (i.e. yeast) to complex multicellular organisms [14]. Furthermore, deletion analysis of human Bcl-2 has defined certain structural domains which can compensate for the growth inhibitory effects of Bax in yeast [15]. The inferences made on growth arrest and rescue have been based solely on co-transformation frequencies of plasmids carrying gene fusions of '*Bcl-2*' (i.e. full-length *Bcl-2*, deletion mutants of *Bcl-2* or a *Bcl-2* family member) and a plasmid carrying a *Bax* fusion. Since a plasmid which constitutively expresses the *Bax* fusion does not furnish any transformants, Bax was deduced to be 'lethal' for yeast [14,15]. The *Bax*/'*Bcl-2*' constructs, which were used to obtain these observations, were all fused to a nuclear targeting signal and domains from the yeast transcription activator Gal4 (commonly used in the yeast two-hybrid system) [14,15,16]. Fusion proteins, bearing a Bcl-2-like protein, were classified as 'rescuing' from the 'lethal' effect of Bax when transformants were obtained in cells coexpressing the *Bax* fusion [14,15].

In order to investigate the effects of Bax and its rescuing partners, Bcl-2 and Bcl-x(L) on *S. cerevisiae* in more detail, we have attempted to express the proteins not as two-hybrid fusions but as discrete entities. The proteins have been expressed in yeast, without any signal peptide, under the control of the inducible *GAL1/GAL10* promoter. Beyond the confirmation of earlier observations [14,15], we find that in yeast cells Bax-mediated cell death (as observed microscopically through loss of cell membrane integrity) and growth inhibition are probably two distinct events. We clearly demonstrate that the functional mitochondrion (the organelle which allows respiration) in Bax-containing cells does not play any part in impeding cell growth. However, the presence of mitochondrial DNA is essential for cell death. We also observe that the C-terminal membrane anchor domain of Bcl-2 plays a definitive role in the rescue of

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respiration-competent Bax expressing cells (i.e. cells which contain intact mitochondrial DNA), while it is seemingly redundant in cells with dysfunctional mitochondria. Our results, obtained from yeast, imply that the oxidoreductive environment of the mitochondrion could be an important element in both apoptosis and its inhibition as seen in mammalian cells.

2. Materials and methods

2.1. Bax expression cassette and expression plasmids

The gene coding for the human Bax protein [17] was chemically synthesized using yeast codon bias. The gene was assembled from complementary deoxyoligonucleotides, each 70 to 80 base long and having 20 bp overlaps, via (i) 5′ to 3′ chain extension followed by (ii) polymerase chain reaction (PCR) amplification. All PCRs have been performed using the Vent polymerase (Biolabs). The complete *Bax* gene (a *Bgl*II–*Xba*I fragment) was constructed in three parts. Each segment was first subcloned in pUC19 and the authenticity of the clones were confirmed by DNA sequencing. A *Sal*I–*Bam*HI *GAL10* promoter fragment [18], the *Bgl*II–*Xba*I *Bax* gene sequence and a *Xba*I–*Sac*I fragment containing the *SUC2* transcription terminator (with or without the 5′ DNA sequence coding for the c-myc epitope EQKLISEEDLNG) were subcloned in the 2μ-vector pDP34 (a *S. cerevisiae–Escherichia coli* shuttle vector) [19]. The episomal plasmid pWG1 was thus obtained. The entire Bax expression cassette was amplified by PCR from pWG1 as a *Bgl*II–*Xho*I fragment. This expression cassette was then subcloned between the *Bam*HI and *Xho*I sites of a yeast integrating vector pPFY7 (a YIp vector with *LEU2* as yeast selection marker) [20], or between the *Bam*HI and *Sal*I sites of pDP34 (a YEp vector containing the complete 2μ sequence and with *URA3* and *dLEU2* as yeast selection marker) and pDP83 (a *CEN-14* YCp vector with *URA3* as yeast selection marker) [19,20]. The plasmids pWG2 (YIp), pWG3 (YEp) and pWG4 (YCp) were thus obtained.

To eliminate the possibility of the c-myc tag having an effect on yeast cell growth, Bax expression plasmids (similar to pWG2, pWG3 and pWG4) without a c-myc tag have been constructed.

Plasmids containing expression cassettes, driven by the constitutive promoters *GAPDHp* (from the yeast glyceraldehyde-3-phosphate dehydrogenase gene) and *CYP1p* (from the yeast cytosolic cyclophilin gene) [19,21], were also constructed. These two expression plasmids are referred to as pDP34/*GAPDHp–Bax* and pDP34/*CYP1p–Bax*, respectively.

2.2. Human Bcl-x(L) expression cassette

The complete coding region of the human Bcl-x(L) protein [22] was cloned from a thymus cDNA library (Clontech) as a *Bgl*II–*Xba*I fragment by PCR. DNA sequencing confirmed the authenticity of the isolated sequence. The Bcl-x(L) expression cassette, under the control of the *GAL10p* and *CYP1p*, was subcloned in pDP34 as described in section 2.1 and is referred to as pDP34/*GAL10p–Bcl-x(L)* and pDP34/*CYP1p–Bcl-x(L)*, respectively.

2.3. Expression cassettes for murine Bcl-2 and murine Bcl-2 variants

The murine Bcl-2 clone [23] was obtained from Professor S. J. Korsmeyer (Washington University, St. Louis). A *Bgl*II–*Xba*I fragment was isolated by PCR in such a way that the 3′-end of the gene coded for the peptide TWIQDNGGWDAFVE. This modified murine Bcl-2 sequence (referred to in this study as mBcl-2) lacks the region which corresponds to the membrane anchor of human Bcl-2 (hBcl-2) [24] but contains the complete BH2 domain as present in hBcl-2. The gene mBcl-2 was subcloned in pUC19 to yield pUC19/*mBcl-2*. The mBcl-2 yeast expression cassette (under the control of *GAL10p* and *CYP1p*) was subcloned in pDP34, in a manner identical to the cloning of Bax and Bcl-x(L) expression cassettes (see sections 2.2 and 2.3).

The plasmid pUC19/*mBcl-2* was digested with *Xba*I and double-stranded linkers (5′-ends of linkers contain *Xba*I overhangs, one of which recreates an *Xba*I site after ligation), coding for the membrane anchor regions of hBcl-2 (KTLLSLALVGACITLGAYLGHK; hma) [5,7] and the yeast mitochondrial outer membrane protein Mas70 (KTAILATVAATGTAIGAYYYYNQ; mma) [7], were ligated unidirectionally. The two new plasmids bear (i) a C-terminal fusion of mBcl-2 and hma and (ii) a C-terminal fusion of mBcl-2 and mma. The expression cassettes containing *Bgl*II–*Xba*I fragments of *mBcl-2–hma* and *mBcl-2–mma* (under the control of *GAL10p* and *CYP1p*) were later subcloned in pDP34 as described above.

2.4. Strains and media

Most of the experiments reported here were performed using the strain HT444 (*MATa leu2–3 leu2–112 his4–519 ura3 lys2*). However, the basic data relating to growth arrest and cell mortality were all confirmed in 3 other strains: SE312 (*MATα leu2 lys2 his4 ura3 pra1 prb1*), TFY2 (*MATα his ura3–52 trp1–285 ace1 LEU2:: YIpCL: CUP1′ Gal*$^+$) [19] and W3116 (*MATa leu2–3 leu2–112 his3 PCR1Δ:: HIS3 ura3–52 lys2 pep4Δ1137*) [20]. An integrated copy of pWG2 in the *LEU2* locus of HT444 (HT444:: pWG2) is referred to as Bi (i.e. the *Bax* integrant).

The yeast cell culture media have been described earlier [25]. The minimal medium is denoted as SD (0.67% yeast nitrogen base, without any amino acids; Difco) whereas rich medium is referred to as YEP (2% bacto-peptone, 1% yeast extract; both from Difco). The supplements added to the SD medium were those required for growth of the specific strain. The carbon sources which have been used for growth are 2% (w/v) glucose (Glu; Fluka) or 2% (w/v) sodium lactate (Lactate; Sigma) or 2% (w/v) galactose (Gal; Fluka).

2.5. Petite formation

Petite mutations were induced in strain Bi (the *Bax* integrant; see above) using ethidium bromide as described by Sherman et al. [25]. A loopful of cells were grown in the dark at 30°C for 36 h, in 10 ml of SD-Glu medium which contained 10 μg/ml ethidium bromide. The cells were spread on YEP-Glu plates to give single colonies. The colonies were replica plated on to YEP plates containing 3% glycerol. Those colonies which grew only on YEP-Glu plates, but not on YEP-glycerol, were classified as petites. The basic observation that petites are growth inhibited but not killed by galactose induction of Bax was confirmed with 3 separate petite segregants. In order to find if mBcl-2 rescued petites, the grande strain Bi:: pDP34/*GAL10p–mBcl-2* was converted to petites as above. Simultaneous growth of 5 individual petite colonies confirmed galactose resistance. Similar observations were made on Bi petites transformed with pDP34/*GAL10p–mBcl-2*.

2.6. Cell staining

Two stains (methylene blue and erythrosine B) were used for identifying cell death (i.e. cells which have lost membrane integrity). The cells were initially grown in SD-Glu (in which case the cells were washed 3 times in sterile water before induction with galactose) or in SD-lactate (in which case the cells were directly induced with galactose).

In order to stain with methylene blue, cells were first resuspended in an appropriate volume of 0.9 M KCl and were then treated with the dye solution at a final concentration of 0.5 g/l [26].

The medium used for staining with erythrosine B [27] contained agarose (2% w/v) in SD, an appropriate carbon source and 7.5 μM erythrosine B (Serva). Cells were first spread on microscope slides and then incubated at 30°C in a humidifying chamber.

Methylene blue stained cells were counted using a haemocytometer.

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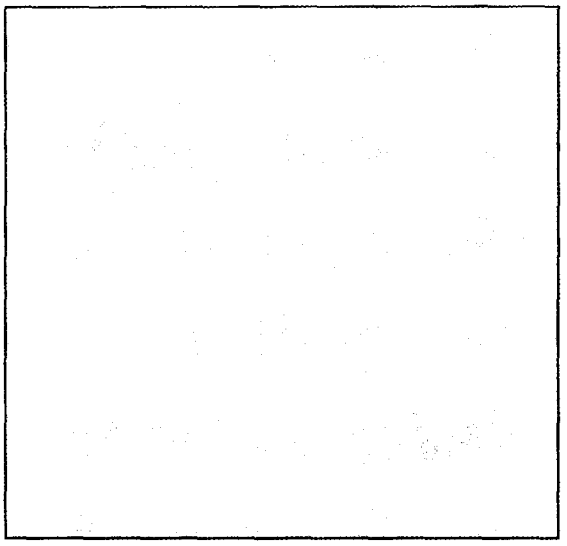
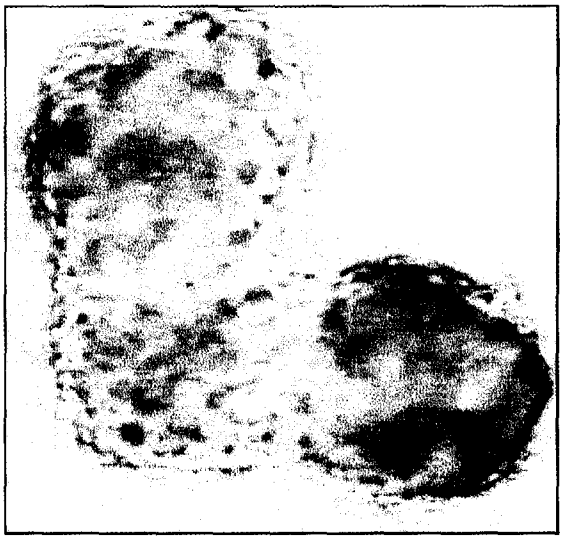
Fig. 1. Erythrosine B and methylene blue staining. In panels (A) and (B) the same cells are visualized after 5 h and 23/24 h. In panel (A), HT444 transformed with pDP34 is shown. Cells divide normally when grown in minimal SD-Gal medium. The photographs are shown in high magnification (400×). Panel (B) shows HT444 transformed with pDP34/*Gal10p-Bax* (see Section 2) when grown in SD-Gal. Cells have been photographed under the same magnification as in panel (A). Some rare cell division is seen after ~5 h. The cells, that do not divide, die soon after this time (pink stained cells). Note the dramatic differences in cell division between the 23/24 h time points of panels (A) and (B). Panel (C) shows (same magnification as above) the fate of a large number of cells (HT444::pDP34/*Gal10p-Bax*), after 12 h of induction, upon methylene blue staining (~90% cells are stained blue).

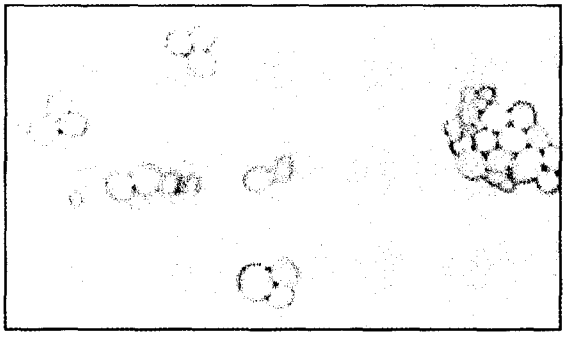
(A) No Bax expression : Erythrosine B staining

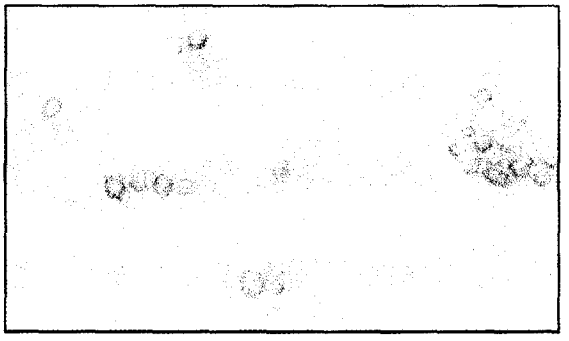




Time = 5 hours **Time = 23 hours**

(B) Bax expression : Erythrosine B staining





Time = 5 hours **Time = 24 hours**

(C) Bax expression : Methylene blue staining

The average and standard error of mean (S.E.M.) was calculated separately for blue (dead) and white ('live') cells in each quadra. The percentage of blue cells were then defined as lying between the extremes (e.g. the highest value would be (Blue + $S.E.M._{blue}$)/ (Blue + $S.E.M._{blue}$ + White − $S.E.M._{white}$). The experiments were repeated and the final range was defined as the overlap of results from different experiments.

2.7. Spot tests

Cells were suspended in water and 5 μl aliquots were spotted on appropriate minimal medium or rich medium plates.

3. Results and discussion

The human Bax protein [17] has been expressed in yeast, using the inducible *GAL10* promoter, under a variety of conditions. The effect of inducing Bax in grande (*Rho*$^+$) and petite (*rho*$^-$) yeast cells, employing different forms of preculture and induction media, is summarized in Table 1. The data were compared with those of the control strain HT444::pPFY7. For this strain, no cell mortality was observed in liquid medium even after prolonged growth in galactose (Gal), lactate or glucose (Glu). It should be noted that pDP34/*GAPDHp–Bax* and pDP34/*CYP1p–Bax* (plasmids which contain Bax expression cassettes under the control of two constitutive promoters *GAPDH* and *CYP1*; see section 2), when transformed into various yeast strains, yielded no transformants.

Our results show that a low level induction of Bax is sufficient to suppress growth and cause some mortality. This is indicated (a) by the accumulation of dead cells in stationary phase even in the absence of galactose (Table 1; row 6, column d), (b) from the levels of mortality in lactate (row 4, column d) and (c) from the growth inhibition observed on resuspension of cells in lactate medium (row 4, column f). It is significant that, although the petites (Bi *rho*$^-$) do not grow at all in rich or minimal galactose media (see rows 1, 2 and 3, column e), no cell mortality is seen even after prolonged incubation (see rows 1, 2 and 3, column c). It is also interesting to note that Bax-expressing grandes divide slowly for 5 h in rich YEP-Gal medium (row 1, column f). However, this does not prevent cell mortality which follows immediately afterwards (row 1, column d). In minimal SD-Gal medium, the grandes fail to show any cell division (row 2, column f) which is in contrast to growth in rich medium. Moreover, there is increased mortality (compare rows 1 and 2, column d). It is clear that, in rich YEP-Gal medium, grande yeast shows less growth inhibition and mortality than in minimal SD medium (compare rows 1 and 2, column d). This is also reflected in the observation that cells, initially grown in YEP-Glu instead of SD-Glu, manifested perceptibly decreased cell growth inhibition and cell death even if cells were later transferred to Bax-inducing minimal SD-Gal medium (Table 1, compare rows 3 and 2, column d).

It has been suggested on the basis of co-transformation experiments that Bax causes cell death, rather than simple growth inhibition [14,15]. In a broad sense, this conclusion is supported by our observations with grande yeast Bi (Table 1, column d). However, we see no cell mortality (as measured by loss of membrane integrity) with the petite yeast Bi *rho*$^-$ (Table 1, column c). We observe a definite delay between the time when cell division is halted (just after induction of Bax) and the onset of membrane breakdown. This is apparent when plated cells, obtained after induction in SD-Gal minimal medium, are examined under the microscope and stained with erythrosine B (Fig. 1; panel B). Although cell growth arrest occurs immediately after Bax induction (i.e. $t > 0$ h), cell death is observed only after 5 h. It seems as if cells which divide are more liable to survive (Fig. 1, panel B, 24 h). However, the majority of cells lose membrane integrity after 12 h (Fig. 1, panel C). In marked contrast to this, control cultures of HT444 grow normally (viz. Fig. 1, panel A, 5 and 23 h).

The murine Bcl-2 protein [23] differs from human Bcl-2 (hBcl-2) [24] most significantly at the C-terminus. It is suspected that the C-terminal membrane-spanning signal anchor sequence of hBcl-2, which is missing from the murine protein, has an important function in preventing apoptosis [5–7]. The gene encoding the published murine Bcl-2 sequence [23] was extended at the 3′-end to incorporate the peptide TWIQDNGGWDAFVE so that the BH2 domain [4,8], as

Table 1
Inhibition of cell growth and cell death (as monitored by methylene blue staining), after induction of Bax in yeast

	Preculture medium	Induction medium	Methylene blue staining after 24 h (%)		Growth in Bax-inducing medium	
			Petite	Grande	Petite	Grande
1	YEP-Glu	YEP-Gal	0*	22–35	No growth	Growth for 2 to 5 h[#]
2	YEP-Glu	SD-Gal	0*	50–70	No growth	No growth
3	SD-Glu	SD-Gal	0*	80–90[∇]	No growth	No growth
4	SD-Glu	SD-lactate	ND	5–10	ND	Slow Growth[#]
5	SD-Lactate	SD-Gal	ND	80–90[∇]	ND	No growth
6	YEP-Glu	To stationary phase	0*	5–10	–	–
	a	b	c	d	e	f

The strain Bi (HT444::pWG2) was grown in liquid medium using a combination of different pre- and main-cultures (see section 2). The media SD-Glu, SD-Gal, YEP-Glu and YEP-Gal have been described in section 2 and, where necessary, were supplemented with the appropriate amino acids and uracil. Although the effects described here pertain to single-copy expression of Bax, no significant differences were observed on expressing the protein from the multicopy episomal plasmid pDP34.

*For the petite, no methylene blue stained cells were seen after 24 h. The number of cells which were examined varied but in all cases exceeded 1000.

[#]The growth rate of Bi in YEP-Gal was similar to the control HT444::pPFY7 over the first few hours (this was followed by comparison of bud appearance in Bi and HT444::pPFY7). In contrast, for strain Bi the growth rate fell from a value of $\mu \cong 0.4$ h^{-1} (in SD-Glu) to <0.05 h^{-1} (in SD-Lactate) while for the control strain it decreased from 0.4 h^{-1} to 0.15 h^{-1}. The measured growth rates were maintained for at least a 24 h period.

[∇]Methylene blue staining indicates loss of membrane integrity. The fact that a cell remains unstained does not indicate that the cell would be able to divide again when Bax expression is repressed. In our experiments, a small percentage of cells (less than 1%) do grow when transferred back to glucose medium, even though 80 to 90% of cells were initially stained.

found in hBcl-2, was complete [24]. This modified gene is referred to as *mBcl-2*. The *mBcl-2* gene has been further extended at the 3′-end to incorporate signal anchor regions from hBcl-2 [5,7] and the yeast outer mitochondrial membrane protein Mas 70 [7]. We have co-expressed the three proteins mBcl-2, mBcl-2–hma (hma being the hBcl-2 membrane anchor) and mBcl-2–mma (mma being the Mas70p membrane anchor) with Bax, in order to find out if the membrane anchor sequence has any influence on the survival of yeast cells.

Table 2 summarizes the effects of co-expressing Bax with Bcl-2-like proteins (from many experiments), under the control of the inducible *GAL10* promoter. Fig. 2 depicts some of these effects more clearly. Expression of human Bcl-x(L) protein in Bi (*Rho*$^+$) rescues Bax poisoned grande cells in both rich and minimal media (Table 2, row 1) but murine Bcl-2 does not (Table 2; row 4). Surprisingly however, mBcl-2 strongly rescues the petite yeast Bi *rho*$^-$ in rich medium but only marginally in SD medium (Table 2; row 5). Although mBcl-2 (without a membrane anchor domain) is incapable of diluting the toxic effects of Bax in grande cells (Table 2; row 4), both mBcl-2–hma and mBcl-2–mma proteins can negate Bax to varying degrees (compare rows 6 and 9). It seems that the membrane anchor from the Mas70 protein is more effective in restoring growth in grande cells than the similar domain from hBcl-2. However, full-length Bcl-x(L) seems to be even more potent in its rescuing capacity than mBcl-2–mma. The undoubted superiority of Bcl-x(L) in rescuing Bax-induced grande cells is emphasized by the growth of Bi in the presence of poorly expressed Bcl-x(L) (from the weakly constitutive promoter *CYP1p*; compare rows 1, 3 with 6, 8 and 9, 10) [21]. However in this context, it is noteworthy that Bcl-x(L) and mBcl-2–mma (two proteins with membrane anchors) are quite alike mBcl-2 (a protein without a membrane anchor) in their capacity to rescue Bi *rho*$^-$ petites in rich medium (compare rows 2 and 7 with row 5). However, petites in minimal medium are unaffected and remain growth arrested in the presence of Bcl-x(L) and mBcl-2–mma.

Table 2
Rescue of yeast cells from Bax-mediated growth-inhibition or cell death by Bcl-x(L), mBcl-2, mBcl-2–hma and mBcl-2–mma proteins

	Expression cassettes	Strain	YEP-Gal	SD-Gal
1	*GAL10p–Bcl-x(L)*	Bi	+++	+++
2	*GAL10p–Bcl-x(L)*	Bi *rho*$^-$	++	–
3	*CYP1p–Bcl-x(L)*	Bi	+++	+++
4	*GAL10p–mBcl-2*	Bi	–	–
5	*GAL10p–mBcl-2*	Bi *rho*$^-$	++	+/–
6	*GAL10p–mBcl-2-mma*	Bi	++	–
7	*GAL10p–mBcl-2-mma*	Bi *rho*$^-$	++	–
8	*CYP1p–mBcl2-mma*	Bi	–	–
9	*GAL10p–mBcl-2-hma*	Bi	+/–	–
10	*CYP1p–mBcl-2-hma*	Bi	–	–
11	–	Bi	–	–
12	–	Bi *rho*$^-$	–	–

The proteins are expressed under the control of *GAL10p* or *CYP1p* from the episomal plasmid pDP34, in yeast strains Bi (the *Bax* integrant) and the petite Bi *rho*$^-$. Rows 11 and 12 show the results for transformants of pDP34, which contain no expression cassette. Cell suspensions were spotted on to YEP-Gal and SD-Gal plates or onto control plates (YEP-Glu and SD-Glu). '–' denotes the complete absence of growth on test plates while confluent growth was observed on control plates. '+++' indicates that there is nearly identical growth on test plates and control plates. '++' implies that there is rescue but less growth on test plates than on controls. Still weaker growth on test plates is depicted as '+', whereas '+/–' denotes growth of point colonies. Fig. 2 visualizes some of the data summarized in this table. It should be noted that the control strain HT444 *rho*$^-$ grows well both in SD-Glu and SD-Gal.

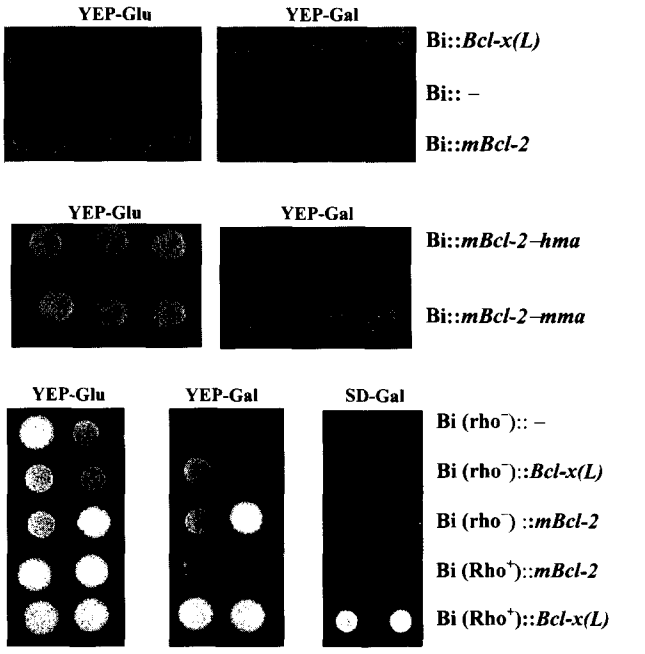


Fig. 2. Induction of Bax expression in the strain Bi (*Bax* integrant), in the presence of Bcl-2-like proteins. Bi was transformed with different episomal plasmids (pDP34-derived) which contained expression cassettes for specific *Bcl-2*-like genes (as indicated). All gene expressions are under the control of the *GAL10* promoter. '–' denotes the transformants of pDP34 (which contain no expression cassette). Equal number of cells were spotted on minimal SD and rich YEP medium plates, in the presence of glucose (Glu) or galactose (Gal), as described in section 2. The control strain HT444 *rho*$^-$ grows well both in SD-Glu and SD-Gal (data not shown).

The overall significance of our results has been highlighted in the data depicted in Tables 1 and 2. Primarily, we find that Bax causes inhibition of cell growth and this can lead to cell death under certain conditions. We show that cell death is subtly dependent on the type of media which is used for growth, before and after Bax induction (Table 1, rows 1–3). The fact that the petite *Bi rho*$^-$ yeast does not suffer lethality in the presence of Bax but is still growth inhibited, suggests that cell death is probably mitochondria-dependent. A lethal mitochondria-dependent pathway could be completely separate from a non-lethal mitochondria-independent growth arrest, or they could be overlapping events. The fact that growth inhibition precedes large scale cell death by at least 5 h indicates that cell death (which is mitochondria-dependent) can be subsequent to the mitochondria-independent pathway (Fig. 3).

It has been shown that a truncated version of hBcl-2 (1–196) does not suppress Bax-mediated growth inhibition in yeast [15]. The mBcl-2 molecule is analogous to hBcl-2 (1–196) and is also ineffective against overcoming Bax's toxicity in grande yeast. However, the mBcl-2 protein rescues petites quite proficiently. Our data also indicate that mBcl-2 can compensate for Bax in grande cells if it is C-terminally linked to the membrane spanning region of the yeast Mas70p. We conclude that the capacity of Bcl-2 to rescue grande cells from the effects of Bax relies on a C-terminal membrane anchor.

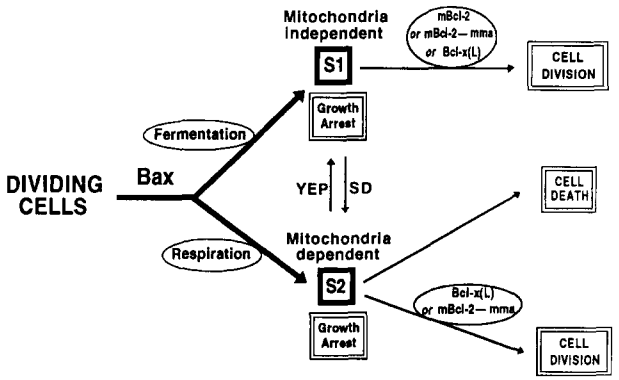


Fig. 3. Possible pathways for Bax action. Bax is induced in dividing cells and causes cells to enter dormant states S1 (mitochondria-independent) and S2 (mitochondria-dependent). Petite cells can go through the S1 pathway only. But grande cells have a choice between S1 and S2 and the two states are probably in equilibrium. In minimal SD medium, the equilibrium shifts towards S2 whereas in rich YEP medium S1 is preferred (as explained in Section 3). However, cell death requires the S2 state. The C-terminal membrane anchor in Bcl-2-like proteins (as in Bcl-x(L) and in mBcl-2–mma) is essential for rescue from S2, while it appears to be redundant in S1 (as in the case of mBcl-2).

Yeast can divide and grow using a respiratory pathway which is dependent on mitochondrial function. Unlike mammalian cells which do not survive in the absence of mitochondria, yeast has a viable alternative. Petite yeast cells can grow merely through fermentation. In cells expressing Bax, growth arrest occurs independent of the mitochondria via an S1 state, as seen in petites (Fig. 3). In grande cells which possess functional mitochondria, growth arrest at S1 could progress to cell death via a respiration dependent S2 state (Fig. 3). Of course, cell death would be facilitated in medium which would encourage greater respiration. Rapidly growing cells encounter a respiratory bottle neck (viz. in YEP medium) [28,29]. Minimal SD medium, which allows only slow cell growth would thus permit more respiration than YEP medium. In this respect it is worth observing that galactose is a less efficient inhibitor of respiration than glucose [30]. Our observations confirm that cell death is indeed more profound under conditions where cells can respire better (viz. in SD medium rather than in YEP medium; Table 1; rows 1–3).

The three proteins mBcl-2, mBcl-2–mma and Bcl-x(L) can rescue petites from the mitochondria-independent S1 state (Fig. 3). A membrane anchor is not essential for rescue through S1. However, rescue from the S2 state needs a membrane anchor domain probably because membrane association of Bcl-2-like proteins is indispensable for overcoming the effects of Bax in the presence of functional mitochondria. The observation that Bcl-x(L) can rescue grande cells in minimal and rich medium (in contrast to mBcl-2–mma which rescues grandes only in rich medium) may imply that small amounts of Bcl-x(L), which are expressed in minimal SD medium, are enough to abrogate the deleterious effects of Bax. Protein expression in SD is known to be poor compared to the amounts produced in rich medium [31]. It is possible that Bcl-x(L) has a much stronger affinity for Bax than Bcl-2. The mechanism by which homodimeric Bax is physically inactivated is believed to occur through formation of Bax-Bcl-2 and Bax-Bcl-x(L) heterodimers [4,8]. It is likely that the amounts of Bcl-x(L) produced in SD, even with the weakly constitutive *CYP1* promoter, are sufficient to nullify the Bax-induced phenotype in grande yeast (i.e. from S2) but not in the petite (i.e. from S1).

The importance of mitochondria in apoptosis is not clearly understood. There have been contradictory findings about its function in apoptosis [32,33]. Unlike multicellular organisms, the yeast *S. cerevisiae* has the unique ability to survive without functional mitochondria and to live by glycolysis alone. Although the classical manifestations of apoptotic cell death (viz. DNA fragmentation) [1–4] may not be apparent in yeast (W.G. and B.C., unpublished data), we feel that the simple eukaryote *S. cerevisiae* could still be used as an organism to elucidate the true role of mitochondria in apoptosis and inhibition of apoptosis.

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