Response of yeast to the regulated expression of proteins in the Bcl-2 family

Peter POLČIC and Michael FORTE[1]

Vollum Institute, Oregon Health & Science University, 3181 S. W. Sam Jackson Park Road, Portland, OR 97239, U.S.A.

The mechanisms by which pro-apoptotic members of the Bcl-2 family of proteins promote the release of mitochondrial factors like cytochrome *c*, subsequently activating the apoptotic cascade, or by which anti-apoptotic family members block this release, are still not understood. When expressed in yeast, Bcl-2 family members act directly upon conserved mitochondrial components that correspond to their apoptotic substrates in mammalian cells. Here we describe a system in which the levels of representative pro- and anti-apoptotic members of the Bcl-2 family can be regulated independently in yeast. Using this system, we have focused on the action of the anti-apoptotic family member Bcl-x_L, and have defined the quantitative relationships that underlie the antagonistic action of this protein on the lethal consequences of expression of the pro-apoptotic family member Bax. This system has also allowed us to demonstrate biochemically that Bcl-x_L has two actions at the level of the mitochondrion. Bcl-x_L is able to inhibit the stable integration of Bax into mitochondrial membranes, as well as hinder the action of Bax that does become stably integrated into these membranes. Taken together, our results suggest that both the functional and biochemical actions of Bcl-x_L may be based on the ability of this molecule to disrupt the interaction of Bax with a resident mitochondrial target that is required for Bax action. Finally, we confirm that VDAC (voltage-dependent anion channel) is not required for the functional responses observed following the expression of either pro- or anti-apoptotic members of the Bcl-2 family.

Key words: apoptosis, Bax, Bcl-x_L, mitochondria, voltage-dependent anion channel (VDAC), yeast.

INTRODUCTION

Although many proteins are known to be involved in the control of PCD (programmed cell death), members of the evolutionarily conserved Bcl-2 family are central regulators. These proteins appear to function primarily by modulating the release of cytochrome *c* and several other protein factors from the mitochondrial intermembrane space into the cytosol, a required step in the activation of the downstream caspases necessary for efficient apoptosis [1], and representing an irreversible commitment point in the apoptotic pathway [2]. The Bcl-2 family includes pro-apoptotic proteins (e.g. Bax, Bak or Bid) that promote the release of mitochondrial factors and anti-apoptotic proteins (e.g. Bcl-x_L and Bcl-2) that prevent the release of these factors [1]. Family members share sequence homology in up to four BH domains (Bcl-2 homology domains) and many also have a C-terminal hydrophobic domain that targets these proteins to membranes. A subset of pro-apoptotic proteins (e.g. Bid and Bad) share homology only in the BH3 domain ('BH3-only' proteins). When cellular location is assessed, anti-apoptotic family members are differentially targeted to intracellular membranes; Bcl-2 is associated with all intracellular membranes, while Bcl-x_L is targeted primarily to the OMM (outer mitochondrial membrane) [3]. In the absence of a death signal, pro-apoptotic family members containing a C-terminal hydrophobic domain can also have diverse cellular locations. Thus pro-apoptotic family members are localized mainly to the OMM (i.e. Bak) or are primarily cytosolic and translocate to mitochondria following a death signal (e.g. Bax) [4,5], although low levels of Bax and Bak may also reside in the endoplasmic reticulum [6].

The precise mechanisms by which pro-apoptotic family members promote the release of these mitochondrial factors, or by which anti-apoptotic family members block this release, have been the subject of much conjecture and are still not understood. Two general models have been developed. The first is based on the observation that both pro- and anti-apoptotic family members are able to form channels in the synthetic phospholipid membranes [7–9]. Consequently, the formation of large Bax oligomers of sufficient size (22 Å) to allow the transfer of proteins like cytochrome *c* across membranes has been observed *in vitro* [10,11] and in ATP-depleted cells [12]. Formation of these oligomeric complexes is prevented by anti-apoptotic Bcl-2 family members by mechanisms that remain to be defined [7,12]. However, it is not yet clear whether, *in vivo*, these large oligomeric complexes of pro-apoptotic molecules like Bax are alone responsible for the release of intermembrane components like cytochrome *c*. A second set of models depends on modulation of the mitochondrial PTP (permeability transition pore) [13,14], or its components, by these proteins. For example, Bax has been proposed to induce the opening of PTP, leading to a massive osmotic swelling of mitochondria, rupture of the outer membrane accompanied by a fall of mitochondrial membrane potential, and the consequent release of proteins from intermembrane space [15,16]. In this scheme, anti-apoptotic family members inhibit PTP opening. Extensions of this general idea have focused on the direct interaction of the VDAC (voltage-dependent anion channel) of the OMM, a proposed integral component of the PTP, with both pro-apoptotic and anti-apoptotic Bcl-2 family members. These models view direct interaction with VDAC as a crucial step in the processes by which Bcl-2 family members exert their effects, although models directly implicating VDAC differ in their mechanistic details [17–26]. Recently, however, a number of reports have failed to implicate the PTP, VDAC or other molecules proposed to participate in the formation of the PTP in the action of Bcl-2 family members or in processes leading to PCD [27–29].

In order to address basic questions on the mechanisms that underlie the action of Bcl-2 family members, we have examined the response of the yeast *Saccharomyces cerevisiae* to the

Abbreviations used: BH domain, Bcl-2 homology domain; OMM, outer mitochondrial membrane; PCD, programmed cell death; PTP, permeability transition pore; VDAC, voltage-dependent anion channel; HA, haemagglutinin.

[1] To whom correspondence should be addressed (e-mail forte@ohsu.edu).

Table 1 Yeast strains used in this study

Strain	Genotype
CML282	*MATa ura3-1*, *ade2-1*, *leu2-3,112*; *his3-11,15*, *trp1-Δ2*, *can1-100*, CMV_p(*tetR-SSN6*)::*LEU2*
CML282/GAL-BAX	CML282 *HIS3*::*GAL1/10-HA-BAX*
CML282/GAL-BAX/TET-BCL-XL*	CML282 *HIS3*::*GAL1/10-HA-BAX* plus pCM252/HA-BCL-XL
CML282/Δpor1/GAL-BAX	CML282 *POR1*::*kanMX4*, *HIS3*::*GAL1/10-HA-BAX*
CML282/Δpor1/GAL-BAX/TET-BCLXL*	CML282 *POR1*::*kanMX4*, *HIS3*::*GAL1/10-HA-BAX* plus pCM252/HA-BCL-XL
CML282/VDAC-HA	CML282 *POR1*::*kanMX4*, *URA3*::*VDAC-HA*
CML282/VDAC-HA/GAL-BAX	CML282 *POR1*::*kanMX4*, *URA3*::*VDAC-HA*, *HIS3*::*GAL1/10-HA-BAX*
CML282/VDAC-HA/GAL-BAX/TET-BCLXL*	CML282 *POR1*::*kanMX4*, *URA3*::*VDAC-HA*, *HIS3*::*GAL1/10-HA-BAX* plus pCM252/HA-BCL-XL
CML282/VDAC-HA/GAL-BAX/pGAL-BAX/TET-BCL-XL	CML282 *POR1*::*kanMX4*, *URA3*::*VDAC-HA*, *HIS3*::*GAL1/10-HA-BAX* plus p426GAL/HA-BAX and pCM252/HA-BCL-XL

* The same strain carrying a plasmid with the point mutation Y101K was used where indicated.

expression of proteins in this family. Although yeast expresses a caspase-like protease which has been implicated in some forms of environmentally induced yeast cell death [30,31], the yeast genome does not encode proteins such as Bcl-2 family members or other proteins involved in programmed cell death in metazoan cells. However, pro-apoptotic Bcl-2 family members (e.g. Bax and Bak) result in cell death when expressed in yeast, and these proteins are targeted to the OMM, as they are in metazoan cells [32–34]. In contrast with mammalian cells, pro-apoptotic molecules like Bax do not require activation when expressed in yeast; Bax is directly targeted to mitochondria in yeast, whereas in mammalian cells Bax is normally localized to cytoplasmic sites and translocated to mitochondria in response to PCD signals. This difference perhaps reflects the absence of molecules forming sites required for cytoplasmic localization in mammalian cells prior to PCD signals, the lack of other endogenous Bcl-2 family members (e.g. anti-apoptotic Bcl-2 family members [11]) or the existence of other pathways (which remain to be defined in any system) that mediate the activation of these molecules in mammalian cells in the absence of BH3-only family members. Bax functions as it does in mammalian cells following targeting to mitochondrial membranes and consequently modulates mitochondrial function in yeast in similar ways, resulting in the release of cytochrome *c*, mitochondrial swelling, alterations of mitochondrial membrane potential, mitochondrial matrix alkalization and cytosolic acidification [32,35–37]. The yeast system therefore allows careful analysis of the mechanisms by which Bax mediates these mitochondrial responses. In contrast, the anti-apoptotic Bcl-2 family member, Bcl-x_L, suppresses Bax-induced cell death when co-expressed in yeast and is also targeted to the OMM [34,38,39]. In summary, all results generated in these studies indicate strongly that Bcl-2 family members act directly upon highly conserved mitochondrial components in yeast that correspond directly to their apoptotic substrates in mammalian cells.

In this report, we describe a system in which the levels of representative pro- and anti-apoptotic members of the Bcl-2 family can be regulated independently in yeast and employ this system to define the quantitative and mechanistic relationships that underlie the antagonistic action of these proteins. By focusing on the action of a specific anti-apoptotic family member, we show that Bcl-x_L promotes cell survival by displacing Bax from mitochondria and preventing the stable integration of Bax into mitochondrial membranes. In addition, Bcl-x_L can inhibit the actions of Bax stably associated with mitochondria. Our results are consistent with the idea that the function of both pro- and anti-apoptotic members of the Bcl-2 family depends on their ability to associate with a common mitochondrial target. Finally, we confirm that VDAC is not required for the functional responses observed following the expression of either pro- or anti-apoptotic members of the Bcl-2 family.

EXPERIMENTAL

Strains, plasmids and growth conditions

The yeast strain used throughout this study was CML282 [*MATa ura3-1*, *ade2-1*, *leu2-3*, *112*; *his3-11*, *15*, *trp1-Δ2*, *can1-100*, CMV_p(*tetR-SSN6*)::*LEU2*], provided kindly by Enrique Herrero (Universitat de Lleida, Lleida, Spain) [40]. These cells express a mutant *tetR-V16* hybrid transactivator that mediates gene induction at *tetO* promoters following binding of the activator to antibiotic (the 'reverse' or 'tet-on' system [40]). Table 1 lists the yeast strains created in the CML282 background and used in this work. Cells were grown on complete media containing the indicated carbon sources and lacking the appropriate amino acids. Yeast were transformed by standard lithium acetate protocols.

To modulate Bax expression levels, sequences encoding the murine Bax protein containing an N-terminal HA (haemagglutinin) tag were placed downstream of the yeast *Gal1/10* promoter and subcloned into the pRS303 integrative vector [41]. The resulting plasmid was cut with *Hin*dIII and linear DNA used for transformation of CML282. Stable integrants (CML282/GAL-BAX) were identified by growth on media lacking histidine and integration at the chromosomal *HIS3* locus confirmed by PCR. For Bax induction, cells were grown to the exponential phase in appropriate selective media containing 2 % glucose; cells were then harvested by centrifugation, washed twice by resuspension in sterile water and transferred to appropriate selective liquid or solid media containing 2 % raffinose and the indicated concentration of galactose. A multi-copy plasmid, pGAL-BAX, was prepared by subcloning the *Sac*I–*Kpn*I fragment from pRS303-GAL-BAX containing GAL1/10-HA-BAX sequences into *Sac*I/*Kpn*I-cleaved pRS426 [42].

To modulate the expression of Bcl-x_L, sequences encoding murine Bcl-x_L were subcloned into centromeric (*CENIV*) vector pCM252 [40] downstream of the tetracycline-responsive *tetO* promoter. The N-terminal HA tag was introduced by PCR using oligonucleotide primers that contain HA sequence and the *Pac*I restriction site (forward, 5′-TAGCTTAATTAAATGTATCCATATGATGTTCCAGATTATGCTTCTCAGAGCAACCGGGAGC-3′, and reverse, 5′-CAGTTTAATTAATAGAGATGGGCTCGTGCC-3′). The PCR product was cleaved with *Pac*I and ligated (TET-BCL-XL). The Y101K mutation (Y101 refers to the residue in the original untagged Bcl-x_L) was introduced into HA-BCL-XL by PCR using the Stratagene Quikchange System (forward primer, 5′-GTTTGAACTGCGGAAACGGAGAGCGTTCAG-3′;

reverse primer, 5′-CTGAACGCTCTCCGTTTCCGCAGTTCA-AAC-3′). Introduction of the mutation was verified by DNA sequencing. For *tetO*-mediated induction of wild-type and Y101K-mutant HA-BCL-XL, cells were grown in glucose-based media as described above, washed and transferred to selective liquid or solid media containing 2 % galactose and the indicated concentration of doxycycline. Expression of Bax was maximally induced by growth in media containing >1 % galactose, and expression of Bcl-x_L was maximally induced by growth in media containing >1 μg/ml doxycycline.

To generate a CML282 derivative containing a deletion of the *POR1* gene, a 1.8-kb *Xho*I/*Pst*I DNA fragment containing sequences 560 bp upstream of the translational initiation site and 369 bp downstream of the translational stop site was inserted into pBluescript (Stratagene). A 0.6-kb *Eco*RV/*Eco*RI fragment within the open reading frame was replaced with a cassette containing a geneticin-(G418) resistance gene (*Eco*RI/*Sma*I fragment of pFA6a-kanMX4 [43]). The resulting plasmid was cleaved with *Bam*HI and *Hin*dIII and used for transformation of CML282 and CML282/GAL-BAX. The derivatives in which the *POR1* gene had been deleted (Δ*por1*) were then selected on plates containing 200 μg/ml G418, generating CML282/Δ*por* and CML282/Δ*por*/GAL-BAX, respectively.

To generate CML282 derivatives in which the wild-type *POR1* gene was replaced with sequences encoding a VDAC protein with a C-terminal HA tag, the tagged VDAC was prepared by PCR using the pBluescript-POR1 plasmid described above as a template and oligonucleotide primers encoding the HA tag (5′-ATACGATCATATGATGTTCCAGATTATGCTTGAACGT-ATATAATCTAATAT-3′ and 5′-TAGCATACATATGGATAAGC-GTCGAAGGACAAAGA-3′). The product of PCR reaction was cleaved with *Nde*I, ligated, and the fusion verified by DNA sequencing. A *Bam*HI/*Hin*dIII fragment containing the VDAC–HA construct was then subcloned into the pRS306 integrative vector [41], linearized by cleavage with *Bbs*I, and transformed into Δ*por1* strains. Stable integrants were selected for growth on medium lacking uracil and integration at the chromosomal *URA3* locus confirmed by PCR, resulting in strains CML282/VDAC-HA and CML282/VDAC-HA/GAL-BAX.

To assess the growth potential of individual strains, cells were grown overnight in media containing 2 % glucose with or without the indicated concentration of doxycycline, diluted to $D_{600} = 0.5$ and 10 μl aliquots of serial 5-fold dilutions spotted on to test plates. Growth was assessed following incubation at 30 °C for 2–3 days. The last four out of five dilutions are shown.

Preparation of yeast extracts, assessment of protein expression by quantitative immunoblotting and cell fractionation

Protein extracts were prepared by alkaline lysis and trichloroacetic acid precipitation [44], separated by electrophoresis on SDS/10 %-polyacrylamide gels and transferred to nitrocellulose membrane by semi-dry electroblotting. Antibodies used for protein detection were a mouse monoclonal antibody directed against the HA epitope (HA1.1; Covance), rabbit polyclonal anti-yVDAC [45], rabbit polyclonal anti-Bcl-$x_{S/L}$ (L-18; Santa Cruz Biotechnology) and rabbit polyclonal anti-AAC2 provided by Dr Martin Klingenberg, Univeristy of Munich, Munich, Germany. Immunoblots of cell extracts or subcellular fractions were subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Promega) and binding was visualized using a chemiluminescent substrate (Pierce). The chemiluminescence was quantified by scanning with a Kodak Digital Image 440 station and analysed using the Kodak Digital Science 1D software package (v. 3.02).

Mitochondria were prepared as described previously [36,46]. Briefly, cells were converted into spheroplasts by enzymic digestion with Zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan) and homogenized with a glass/Teflon homogenizer. The homogenate was centrifuged at 3000 ***g***, and the resulting supernatant centrifuged at 10000 ***g*** to pellet heavy membranes enriched in mitochondria. The supernatant from this centrifugation was re-centrifuged at 10000 ***g*** and subsequent supernatant containing the cytosol loaded on to SDS/polyacrylamide gels. The heavy membrane fraction was washed twice with homogenization buffer prior to separation on SDS/10 %-polyacryamide gels.

For alkali treatment, the mitochondrial pellet was resuspended at 0.25 mg of protein/ml in freshly prepared 0.1 M sodium carbonate, pH 11.5, and incubated on ice for 30 min. Membranes were then pelleted by centrifugation at 150000 ***g***, whereas proteins removed from mitochondria by alkali treatment were precipitated from the resulting supernatant by addition of trichloroacetic acid to 7 %, centrifuged and washed with acetone. Both alkali-extracted proteins and membrane fractions were analysed by SDS/PAGE and Western blotting.

RESULTS

Response of yeast to variations in the level of Bax

In order to extend the analysis of Bcl-2 family members in yeast, we established a system in which the level of expression of members of the Bcl-2 family can be regulated independently in a common yeast strain, CML282. This strain constitutively expresses a modified tetR protein that mediates the expression of target genes placed downstream of *tetO* sequences in response to tetracycline or its analogues (‘reverse’ or ‘tet-on’ system [40]). To regulate the expression of a representative pro-apoptotic family member, sequences encoding the Bax protein containing an N-terminal HA tag were placed downstream of the *Gal1/10* promoter and integrated into the genome at the *HIS3* locus (CML282/GAL-BAX). Previous studies have demonstrated that protein expression mediated by the *Gal1/10* promoter is proportional to the amount of galactose present in the growth medium [47]. To regulate the expression of a representative anti-apoptotic family member, sequences encoding the Bcl-x_L protein also containing an N-terminal HA tag were placed in a *CEN* plasmid downstream of the *tetO* promoter (TET-BCL-XL). Characterization of this system has demonstrated that the level of protein expression mediated by the *tetO* promoter in CML282 is proportional to the amount of doxycycline present in the growth medium [40]. Finally, CML282 was further modified by replacement of the wild-type gene encoding the major VDAC isoform expressed in yeast (*POR1*) with sequences encoding VDAC molecules containing a C-terminal HA tag under the control of the native *POR1* promoter. As shown in Figure 1(a), the resulting strain expressed levels of HA-tagged VDAC similar to that generated by the wild-type *POR1* gene. Figure 1(b) demonstrates that HA-modified forms of VDAC functioned as unmodified VDAC proteins, since VDAC–HA could fully complement growth defects observed in Δ*por1* strains when grown on non-fermentable carbon sources at restrictive temperatures [45]. Thus in the strain containing the HA-modified versions of Bax, Bcl-x_L and VDAC, the levels of expression of Bax and Bcl-x_L were regulated independently by the concentration of galactose or doxycycline present in the growth media, respectively. The common HA tag in each of these proteins allowed quantitative assessment of the levels of individual Bcl-2 family members required for each functional response (i.e. cell death or survival) relative to each other and relative to a common standard in the OMM, VDAC.

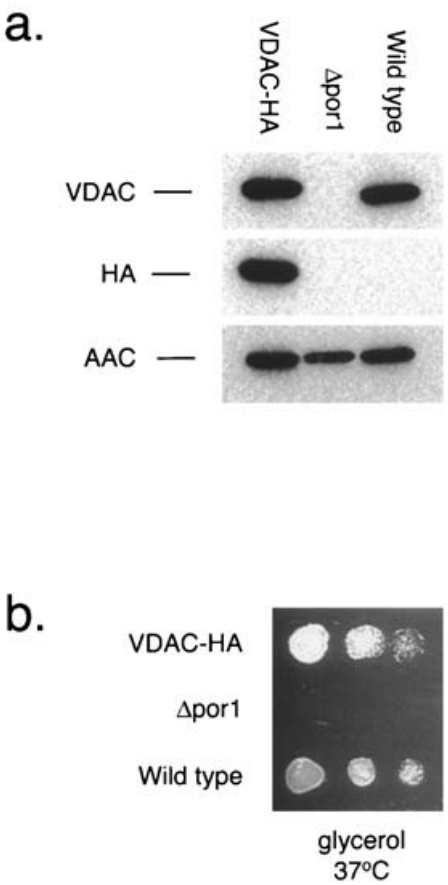

Figure 1 HA-tagged VDAC is expressed at similar levels to and functions as wild-type VDAC

(**a**) Expression of VDAC in CML282 in which the endogenous *POR1* gene has been replaced with the VDAC–HA construct (see the Experimental section) compared with wild-type levels of expression in CML282 (wild-type) and CML282 lacking *POR1* (Δ*por1*). Immunoblots were probed with antibodies directed to yeast VDAC, the HA epitope and yeast AAC2 protein. (**b**) VDAC–HA complements phenotypes resulting from the deletion of *POR1*. Cells in which the *POR1* gene has been deleted are unable to grow on non-fermentable carbon sources at 37 °C [45]. 5-fold serial dilutions of suspensions from strains containing the VDAC–HA construct, lacking *POR1* (Δ*por1*) and CML282 (wild-type) were tested for growth at 37 °C on media containing 2 % glycerol as the sole carbon source. Growth was assessed after 4 days.

To assess the level of Bax protein required to kill cells, we assessed cell viability following serial dilution of strains containing GAL–Bax on to plates containing increasing concentrations of galactose. As expected, the amount of Bax protein expressed was proportional to the amount of galactose present in the growth media (Figures 2a–2c). Yeast viability was dramatically compromised under conditions that mediated maximum levels of Bax expression (>1 % galactose; Figure 2d). When the levels of Bax protein were reduced by growth in medium containing lower levels of galactose, we found that Bax expression mediated by 0.1 % galactose killed cells as effectively as higher concentrations (Figure 2d). At lower levels of galactose (≈0.05 %), Bax expression was still evident (Figure 2b), yet this level of expression had a negligible impact on yeast viability (Figure 2d). Quantitative immunoblotting using the common HA antibody indicated that the minimum level of Bax required to kill cells (i.e. as mediated by 0.1 % galactose) was roughly 200-fold less than the level of VDAC present in the OMM (Figure 2c).

Response of yeast to variations in the level of Bax and Bcl-x_L

We next assessed the level of Bcl-x_L required to rescue cells from Bax-mediated cell killing in CML282/GAL-BAX cells transformed with plasmids that mediate the expression of Bcl-x_L (TET-BCL-XL) in response to the addition of doxycycline to the growth medium. As expected, the amount of Bcl-x_L protein expressed was proportional to the amount of doxycycline present in the growth medium (Figures 3a and 3b); maximal expression was induced by 0.5 μg/ml doxycycline. The level of Bcl-x_L expressed at 0.5 μg/ml doxycycline was sufficient to completely rescue cells from the toxicity associated with maximal Bax expression (>1 % galactose; results not shown). We then determined the minimum level of Bcl-x_L required to protect cells from the effects of Bax by assessing viability following serial dilution on to plates containing different concentrations of both galactose and doxycycline. Expression of Bcl-x_L as mediated by 0.1 μg/ml doxycycline was sufficient to rescue cells from Bax-induced cell killing at all concentrations of galactose tested (Figure 3c).

To test further the functional relationship between the levels of Bax and Bcl-x_L, we transformed CML282/GAL-BAX cells with multi-copy plasmids containing a GAL–Bax construct similar to that integrated at the *HIS3* locus. In these cells, galactose induction of Bax expression results in roughly 4-fold higher levels of Bax than observed in cells containing integrated GAL–Bax constructs alone (Figure 3d). Again, Bcl-x_L expression as mediated by 0.1 μg/ml doxycycline was required to rescue these cells from Bax-mediated cell killing (Figure 3e).

Since the signals generated by Bcl-x_L and VDAC on immunoblotting with the common HA antibody overlap, we used quantitative immunoblotting to compare the levels of Bcl-x_L generated by different concentrations of doxycycline (Figure 3a) with the amount of Bax driven at 2 % galactose in the strain expressing non-tagged VDAC, and then we extrapolated to VDAC indirectly from the Bax/VDAC ratio at this concentration of galactose (Figure 3b). Under these conditions, reflecting maximal Bax expression, the level of Bcl-x_L that was required to rescue cells (i.e. at 0.1 μg/ml doxycycline) was roughly equivalent to the level of Bax and corresponded to levels 20-fold lower than the level of VDAC. However, the results also indicated that the same level of Bcl-x_L was required to rescue cells at the lowest level of Bax required for killing (0.1 % galactose); this concentration of galactose mediates the expression of 10-fold lower levels of Bax. Furthermore, the level of Bcl-x_L required for rescue did not change even if Bax levels were increased 30–40-fold. By extension then, the level of Bcl-x_L required to rescue cells from Bax-mediated killing was roughly 20-fold lower than the level of VDAC, irrespective of the levels of Bax expressed.

Results indicating that the same level of Bcl-x_L is required to rescue cells from Bax killing, even if Bax levels are dramatically changed, make it unlikely that heterodimerization of Bcl-x_L with Bax plays a role in the ability of Bcl-x_L to rescue cells. Consistent with this idea, TET-mediated expression of a Bcl-x_L protein containing mutation of amino acid 101 from tyrosine to lysine (Y101K), which blocks the ability of Bcl-x_L to heterodimerize with Bax [39], demonstrated that the levels of mutant Bcl-x_L required to rescue cells from Bax-mediated cell killing were identical with those observed for wild-type Bcl-x_L at all concentrations of galactose tested (results not shown).

Bcl-x_L inhibits productive Bax interaction with mitochondrial membranes

We examined the cellular localization of both Bax and Bcl-x_L by subcellular fractionation (Figure 4). Bax was targeted largely to

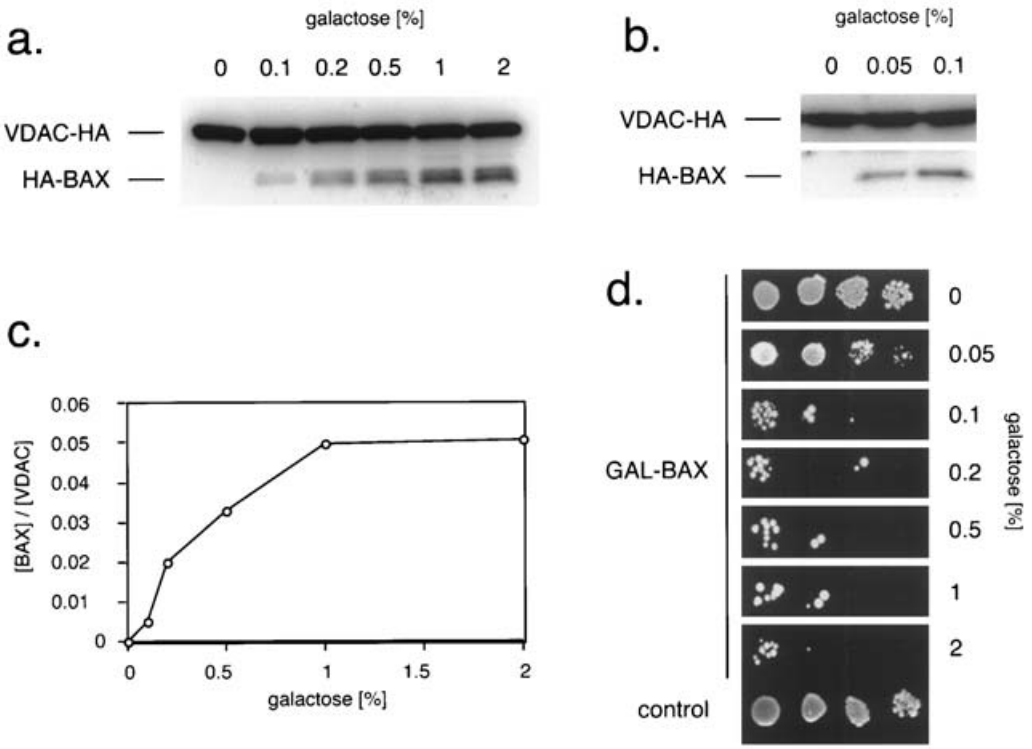

Figure 2 Response of yeast to variations in the level of Bax

CML282/VDAC-HA cells containing an integrated GAL–Bax construct were grown in media containing 2 % raffinose and varying levels of galactose. Expression of Bax relative to VDAC and growth responses were assessed. (**a** and **b**) Extracts were prepared from cells following growth for 24 h in media with varying levels of galactose. Expression of Bax and VDAC was assessed on immunoblots probed with antibodies directed to the HA epitope present in both VDAC and Bax. Expression of Bax induced by 0.05 % galactose is shown on the longer exposure of the blot in (**b**). (**c**) Quantification of signals on immunoblots by procedures outlined in the Experimental section. The ratio of Bax to VDAC is shown as a function of concentration of galactose. (**d**) Growth of CML282 cells containing GAL–Bax construct in response to varying concentrations of galactose. Growth of CML282 (control) is shown on media containing 2 % galactose. 5-fold serial dilutions of cell suspensions were spotted on to plates containing the indicated concentrations of galactose and growth was assessed after 3 days.

mitochondria in the absence of Bcl-x_L. Furthermore, under these conditions, mitochondrially targeted Bax was completely resistant to alkali treatment, indicating that it is stably integrated into mitochondrial membranes. Subcellular fractionation also demonstrated that on co-expression of Bcl-x_L to the levels that protect cells from Bax-mediated killing (on 0.1 and 2 μg/ml doxycycline), significant cytoplasmic accumulation of Bax was observed. Moreover, while Bax expressed in the absence of Bcl-x_L is primarily associated with mitochondria in an alkali-resistant form, significant amounts of Bax could be extracted from mitochondria by alkali treatment on co-expression of Bcl-x_L; roughly half of the Bax expressed in the presence of Bcl-x_L can be extracted by alkali, while the remaining Bax appears stably associated with mitochondria. Similar results were obtained with cells co-expressing mutant Bcl-x_L^{Y101K} (results not shown), indicating that the ability of Bcl-x_L to form heterodimers does not affect subcellular integration of Bax into mitochondrial membrane. In all cases, Bcl-x_L was primarily localized to mitochondria in an alkali-resistant manner.

Deletion of VDAC does not change sensitivity of cells to Bax and Bcl-x_L

We next assessed whether the absence of the outer mitochondrial channel, VDAC, shifts in any way the level of Bax required to kill yeast cells or the level of Bcl-x_L required to rescue cells from Bax-mediated killing. Derivatives of CML282/GAL-BAX were generated in which the gene encoding the VDAC1 protein in yeast, *POR1*, had been deleted by homologous recombination. As was the case in wild-type cells, the amount of Bax and Bcl-x_L expressed was again proportional to the amount of galactose and doxycycline, respectively, present in the growth medium (results not shown) and the levels of Bax expression mediated by 0.1 % galactose were again sufficient to kill cells as effectively as higher concentrations (results not shown). These results confirm previous studies demonstrating that VDAC is not required for Bax-mediated killing [36,48] and furthermore that the levels of Bax required for cell killing in Δ*por1* cells were similar to that required in wild-type cells.

To extend these studies, we determined whether the level of Bcl-x_L protein required to rescue the Δ*por1* strain from Bax-mediated killing was similar to that observed in wild-type cells. CML282/GAL-BAX/Δ*por1* cells were transformed with plasmids described above that mediate expression of Bcl-x_L following addition of doxycycline to the growth medium. As was observed in wild-type cells, the amount of Bcl-x_L protein expressed was proportional to the amount of doxycycline present in the growth medium (Figures 5a and 5b). Cell suspensions were then serially diluted and spotted to plates with varying concentrations of galactose and doxycycline. At all concentrations of galactose tested, the same concentration of doxycycline (0.1 μg/ml) was

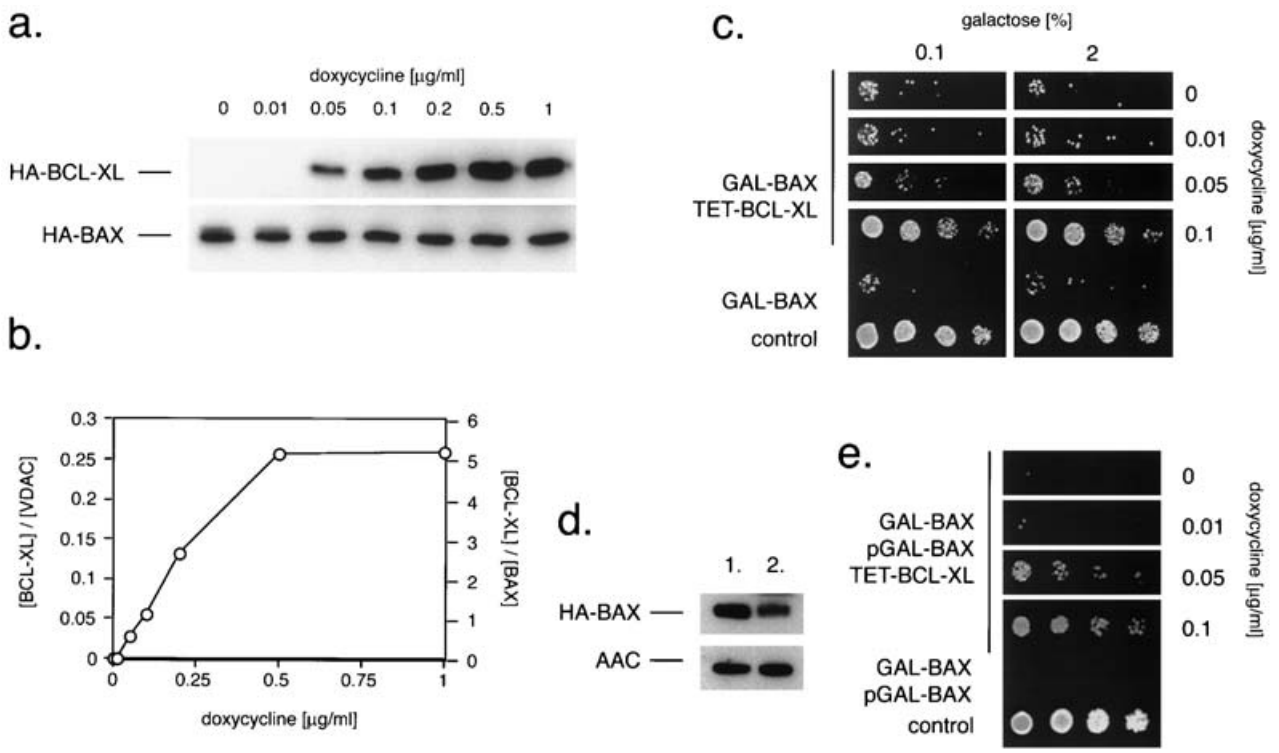

Figure 3 Response of yeast to variations in the level of Bax and Bcl-x_L

CML282/GAL-BAX cells harbouring the TET-BCL-XL plasmid were grown in media containing varying concentrations of galactose and doxycycline. Expression of Bax and Bcl-x_L, and growth responses were assessed. (**a**) Extracts were prepared from cells following growth for 24 h in medium containing 2 % galactose and varying concentrations of doxycycline. Expression of Bcl-x_L and Bax was assessed on immunoblots probed with antibodies directed to the HA epitope. (**b**) Quantification of signals on immunoblots by procedures outlined in the Experimental section. The Bcl-x_L/VDAC ratio was determined by extrapolation from the Bcl-x_L/Bax ratio and the data shown in Figure 2(b). The ratio of Bcl-x_L to both VDAC (left-hand axis) and Bax (right-hand axis) is shown as a function of doxycycline concentration. (**c**) Growth of yeast cells containing Bax and TET-BCL-XL (GAL–BAX, TET-BCL-XL) in response to varying concentrations of galactose and doxycycline. Growth of controls strains containing GAL–Bax and TET vector (GAL–BAX) and wild-type CML282 containing TET vector (control) are shown at the highest concentration of doxycyline. 5-fold serial dilutions of cell suspensions were spotted on to plates containing the indicated concentrations of galactose and doxycycline, and growth was assessed after 3 days. (**d**) CML282/GAL-BAX cells were co-transformed with pGAL-BAX and TET-BCL-XL plasmids. Extracts were prepared from cells following growth for 24 h in medium containing 2 % galactose. Expression of Bax was assessed on immunoblots probed with antibodies directed to the HA epitope. Blots were also probed with antibodies directed to the yeast AAC2 protein. Lane 1, extracts from CML282/GAL-BAX cells harbouring the pGAL-BAX plasmid. Lane 2, extracts from CML282/GAL-BAX cells harbouring vector alone. Quantification of the signals indicates that CML282/GAL-BAX cells harbouring the pGAL-BAX plasmid express 3–4-fold-higher levels of Bax relative to CML282/GAL-BAX cells. (**e**) Growth of yeast cells containing Bax, TET-BCL-XL and pGAL-BAX (GAL–BAX, pGAL-BAX, TET-BCL-XL) in response to medium containing 2 % galactose and various concentrations of doxycycline. Growth of control strains containing GAL–Bax, pGAL-BAX and TET vector (GAL–BAX, pGAL-BAX) and wild-type CML282 containing GAL and TET vectors (control) is shown at the highest concentration of doxycycline. 5-fold serial dilutions of cell suspensions were spotted on to plates containing the indicated concentrations of doxycycline, and growth was assessed after 3 days.

required to rescue cells as was observed in wild-type cells (Figure 5c). Furthermore, quantitative immunoblotting as described above indicated that the same level of Bcl-x_L proteins found to counteract the functional effects of Bax expression on cell viability in wild-type cells is required to generate these effects in Δ*por* cells (Figures 5b and 5c).

DISCUSSION

The means by which pro-apoptotic Bcl-2 family members promote the release of mitochondrial factors, which initiate PCD processes, or by which anti-apoptotic family members block this release, has been the subject of much conjecture and is still not understood. We, and others, have examined the response of the yeast, *Saccharomyces cerevisiae*, to the expression of proteins in this family in order to address basic questions on the mechanisms that underlie the action of Bcl-2 family members [32–37,48–50]. These earlier studies demonstrate that Bcl-2 family members are likely to act upon highly conserved mitochondrial components in yeast that correspond directly to their apoptotic substrates in mammalian cells, generating similar, if not identical, biochemical and physiological responses.

Previous genetic studies utilizing the yeast system to examine the function of Bcl-2 family members have often produced inconsistent results; e.g. alternately showing that yeast genes encoding inner membrane adenine nucleotide transporters [49,51,52] and the outer membrane channel VDAC [18,36,48,53] are required or not required for the response of yeast cells to the expression of pro-apoptotic Bcl-2 family members. In earlier studies, we have demonstrated that the response of yeast to the expression of a representative pro-apoptotic Bcl-2 family member, Bax, was not dependent on a variety of genes encoding proteins (e.g. VDAC) required for wild-type mitochondrial function. To extend these studies, we have established a system in which the level of expression of both pro- and anti-apoptotic members of the Bcl-2 family can be regulated independently in a set of strains with a common genetic background, eliminating genetic background differences as a potential source of the inconsistent results noted above. The strains used in this study contain HA-modified

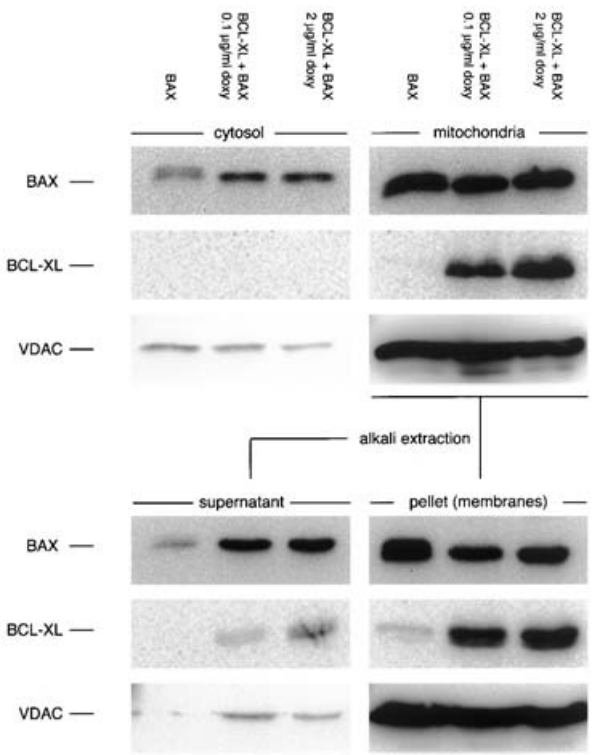

Figure 4 Subcellular localization of Bax and Bcl-x_L

CML282/GAL-BAX cells harbouring TET-BCL-XL plasmid or TET vector were grown in media containing 2% galactose supplemented with 0.1 and 2 μg/ml doxycycline (doxy), converted into spheroplasts, suspended in isotonic buffer, and homogenized. Homogenates were separated into soluble cytosolic and heavy-membrane fractions enriched in mitochondria by differential centrifugation. Protein (50 μg) of each fraction was separated by SDS/PAGE (upper panel). Mitochondrial fractions were subsequently subjected to extraction with 0.1 M sodium carbonate, pH 11.5, and membranes were pelleted by centrifugation. Supernatants and pellets generated from 50 μg of total mitochondrial protein were separated by SDS/PAGE. Immunoblots were probed with antibodies directed to the HA epitope and Bcl-x_L and yeast VDAC1.

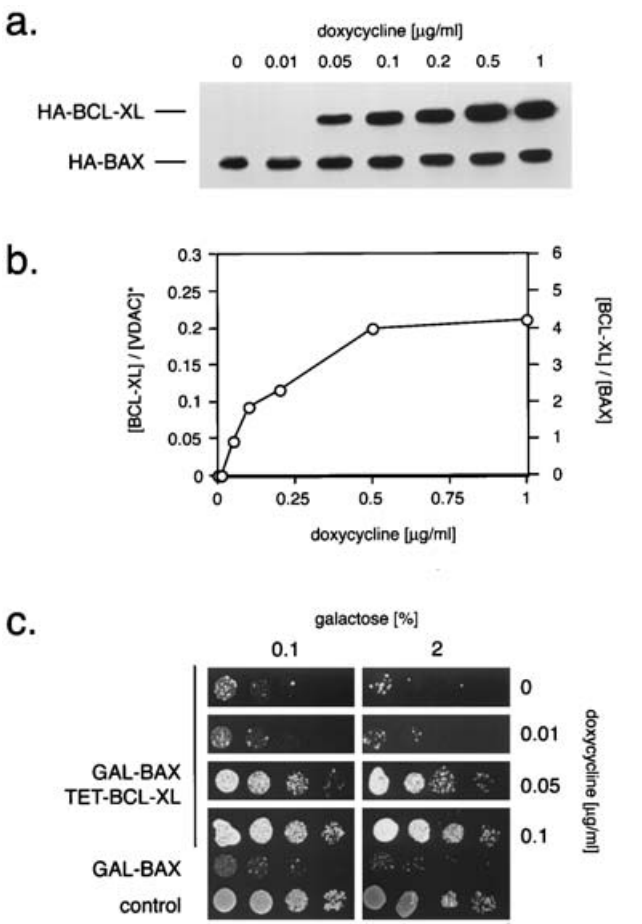

Figure 5 Response of yeast lacking VDAC to variations in the level of Bax and Bcl-x_L

CML282/Δ*por1* cells containing the GAL–Bax construct and harbouring the TET-BCL-XL plasmid were cultivated in media containing varying concentrations of galactose and doxycycline. Expression of Bcl-x_L and Bax, and growth responses were assessed. (**a**) Extracts were prepared from cells following growth for 24 h in medium containing 2% galactose and the indicated concentrations of doxycycline. Expression of Bcl-x_L and Bax was assessed on immunoblots probed with antibodies directed to the HA epitope. (**b**) Quantification of signals on immunoblots by procedures outlined in the Experimental and Results sections. The level of Bcl-x_L relative to Bax (right-hand axis) and to the level of VDAC expressed in the wild-type cells (left-hand axis) is shown as a function of doxycycline concentration. (**c**) Growth of CML282/Δ*por* cells containing GAL–Bax and TET-BCL-XL (GAL–BAX, TET-BCL-XL) in response to media containing the indicated concentrations of galactose and doxycycline. Growth of control strains containing GAL–Bax and TET vector (GAL–BAX) and CML282/Δ*por1* cells containing TET vector (control) is shown at the highest concentration of doxycyline. Serial dilutions (5-fold) of cell suspensions were spotted.

versions of Bax, Bcl-x_L and VDAC, allowing the level of expression of Bax and Bcl-x_L to be regulated independently by the concentration of galactose or doxycycline present in the growth medium, respectively, and functional responses directly compared with quantitative assessment of the levels of individual Bcl-2 family members, relative to each other and to a common standard in the OMM.

Quantitative immunoblotting using the common HA antibody indicated that the minimum level of Bax required to kill cells (i.e. as mediated by 0.1% galactose) was roughly 200-fold less that the level of VDAC present in the OMM, demonstrating that the functional effects of Bax expression do not require levels of Bax equivalent to the levels of VDAC present in the OMM. Lower levels of galactose (i.e. 0.05%) had negligible effects on yeast viability despite low, yet significant, levels of Bax expression. Expression of Bcl-x_L as mediated by 0.1 μg/ml doxycycline was sufficient to completely rescue cells from Bax-induced cell killing at all levels of Bax expression. Indeed, following transformation with plasmids that mediate even higher levels of expression of Bax in response to galactose, the same level of Bcl-x_L expression (i.e. as mediated by 0.1 μg/ml doxycycline) was sufficient to abrogate the effects of Bax expression on cell viability. Thus the level of Bcl-x_L required for rescue does not change even if Bax levels are varied over a 30–40-fold range. These quantitative results make it unlikely that heterodimerization of Bcl-x_L with Bax plays a role in the ability of Bcl-x_L to rescue cells. Consistent with this conclusion, expression of mutant Bcl-x_L proteins unable to heterodimerize with Bax [39] demonstrated that the levels of mutant Bcl-x_L required to rescue cells from Bax-mediated cell killing was identical to that observed for wild-type Bcl-x_L. These results confirm a number of studies that have demonstrated that the formation of inactive Bax/Bcl-x_L heterodimers is likely not to be the mechanistic basis for the ability of anti-apoptotic family members like Bcl-x_L to promote cell survival in response to PCD signals in either yeast or mammalian cells [39,50].

To examine potential mechanisms that might underlie the quantitative relationship between the levels of Bax and Bcl-x_L

and the consequent growth response of yeast, we assessed mitochondrial targeting and stability of integration in association with mitochondrial membranes of both Bax and Bcl-x_L. In the absence of Bcl-x_L, Bax is targeted preferentially to mitochondria, as has been observed in previous studies [35,36,48], and virtually all expressed Bax is fully integrated into the mitochondrial membrane, as indicated by resistance to alkali extraction; this form of Bax is probably responsible for the cytotoxic effect in yeast. Bcl-x_L is also targeted preferentially to mitochondria, as it is in mammalian cells [3], even on co-expression of Bax. Interestingly, a related anti-apoptotic family member, Bcl-2, is distributed to a variety of intracellular membranes in both mammalian cells and yeast [3,54]. Similar to earlier observations [34], significant cytosolic accumulation of Bax was observed when Bcl-x_L was co-expressed with Bax, at the lowest levels of Bcl-x_L that completely rescue cells. Even under these conditions, however, significant levels of Bax protein ($\approx$50%) were integrated into mitochondrial membranes in an alkali-resistant manner. Although it is technically difficult to make accurate quantitative conclusions from fractionation and alkali-extraction data, it is likely that the amount of Bax stably integrated into mitochondrial membranes on co-expression of Bcl-x_L was several-fold higher than the amount of total Bax necessary for killing (e.g. on expression mediated by 0.1% galactose). Since at this level, Bcl-x_L fully maintains the viability of cells expressing Bax (at any level), Bcl-x_L not only inhibits the association of Bax with mitochondrial membranes, resulting in cytosolic displacement and the failure to stably integrate into these membranes, but can also protect cells by preventing membrane-integrated Bax from acting to produce cytotoxic effects. Furthermore, our results suggest that Bcl-x_L does not mediate cell survival through direct interaction with Bax itself, since the same amount of Bcl-x_L can protect cells regardless of the amount of Bax present.

Based on these data we propose a working model, in which Bax and Bcl-x_L share a common target in the OMM. This hypothetical target preferentially associates with Bcl-x_L. In the absence of Bcl-x_L, association of Bax with the target results in permeabilization of membrane and cell death. When Bcl-x_L is co-expressed, Bcl-x_L associates with the hypothetical target and prevents or blocks its interaction with Bax. Under this scenario, occupation of a critical proportion of these targets by Bax is required to alter growth potential, since growth at low levels of galactose (e.g. 0.05%) has little consequence on yeast cell viability. By extension, our results would suggest that only when all or a majority of the critical targets are occupied with Bcl-x_L is the cell protected from the effects of Bax, again when expressed at any level. Displacement of Bax from mitochondria and the lower efficiency of its stable integration into mitochondrial membranes may be the consequence of Bax's inability to access this target in the presence of Bcl-x_L. A similar phenomenon has been observed in the case of VDAC, since newly imported VDAC is unable to stably integrate into OMMs in the absence of pre-existing VDAC complexes [55].

Clearly, the identity of the components forming the target defined in these studies is an important issue. Our results are compatible with the notion that the mitochondrial target of these Bcl-2 family members is formed by either a protein or complex of proteins, or by specific mitochondrial lipids like cardiolipin [56]. Models invoking association with a specific mitochondrial protein would be consistent with the recent finding that Bax failed to form oligomeric complexes in the membranes in the absence of mitochondrial proteins [11], suggesting that the critical target may, in part, be composed of proteins.

Of the possible mitochondrial targets that have been proposed for the interaction with Bcl-2 family members, a number of studies in both yeast and mammalian cells have implicated direct interaction of VDAC as being required for Bax-mediated toxicity or the ability of Bcl-x_L to abrogate Bax-mediated effects [23,25,26]. While the yeast genome contains two genes that encode VDAC isoforms, *POR1* and *POR2* [45], previous characterization of the proteins encoded by each gene has indicated that the major functional VDAC present in the yeast OMM is encoded by the *POR1* gene [45,57]. Here we have used the system developed to carefully examine the sensitivity of yeast lacking the *POR1* gene to the expression of Bax and Bcl-x_L. The results confirm that VDAC is not required for the action of Bcl-2 family members in yeast, nor does it determine the sensitivity of yeast to the functional effects of the expression of these molecules. Thus VDAC is probably not a component of the Bax/Bcl-x_L target in the OMM. Our results are also in contrast with previously published work suggesting that yeast cells missing the *POR1* gene have a marginally increased sensitivity to Bax expression [58]. Finally, it is worth noting that VDAC has been demonstrated to be dispensable for the formation of a novel Bax-dependent OMM channel that has recently been characterized in yeast mitochondria by patch-clamping [53].

Extension of these results to mammalian cells would predict that, in the absence of a PCD signal, anti-apoptotic family members residing in the OMM would preferentially associate with the analogous mitochondrial target. Following a PCD signal, translocation of pro-apoptotic molecules like Bax to the mitochondrion, coupled with the action of BH3-only molecules on both Bax and pro-apoptotic family members that normally reside in the OMM, like Bak, lead to the effective association of these molecules with this target. The conformational changes associated with the activation of pro-apoptotic family members, perhaps oligomerization [12,59–62], coupled with or driven by their interaction with the target, would then initiate the release of intermembrane-space components, like cytchrome *c*, and the PCD cascade.

Experiments leading to the development of the model outlined above would have been difficult to conduct in mammalian cells, and point out the advantages of the yeast system for uncovering basic mechanisms that underlie the function of proteins in the Bcl-2 family. The goal of future studies will be to use the biochemical and genetic approaches possible in this organism to test further the implications of this model and to define the molecular nature of the target suggested by our results.

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REFERENCES

1 Gross, A., McDonnell, J. M. and Korsmeyer, S. J. (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev. **13**, 1899–1911
2 Wang, X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev. **15**, 2922–2933
3 Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R. and Borner, C. (2003) Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. J. Cell Biol. **160**, 53–64
4 Hsu, Y. T., Wolter, K. G. and Youle, R. J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. U.S.A. **94**, 3668–3672
5 Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G. and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. **139**, 1281–1292
6 Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T. and Korsmeyer, S. J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science **300**, 135–139
7 Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazzei, G. et al. (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science **277**, 370–372

8 Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M. and Reed, J. C. (1997) Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U.S.A. **94**, 5113–5118

9 Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M. and Thompson, C. B. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature (London) **385**, 353–357

10 Saito, M., Korsmeyer, S. J. and Schlesinger, P. H. (2000) BAX-dependent transport of cytochrome *c* reconstituted in pure liposomes. Nat. Cell Biol. **2**, 553–555

11 Roucou, X., Montessuit, S., Antonsson, B. and Martinou, J. C. (2002) Bax oligomerization in mitochondrial membranes requires tBid and a mitochondrial protein. Biochem. J. **368**, 915–921

12 Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A. and Saikumar, P. (2001) Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. **276**, 18361–18374

13 Zoratti, M. and Szabo, I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta **1241**, 139–176

14 Bernardi, P., Colonna, R., Costantini, P., Eriksson, O., Fontaine, E., Ichas, F., Massari, S., Nicolli, A., Petronilli, V. and Scorrano, L. (1998) The mitochondrial permeability transition. Biofactors **8**, 273–281

15 Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z. H., Reed, J. C. and Kroemer, G. (1998) The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. J. Exp. Med. **187**, 1261–1271

16 Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A. and Herman, B. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta **1366**, 177–196

17 Shimizu, S., Narita, M. and Tsujimoto, Y. (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature (London) **399**, 483–487

18 Shimizu, S., Shinohara, Y. and Tsujimoto, Y. (2000) Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene **19**, 4309–4318

19 Shimizu, S., Konishi, A., Kodama, T. and Tsujimoto, Y. (2000) BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc. Natl. Acad. Sci. U.S.A. **97**, 3100–3105

20 Shimizu, S., Ide, T., Yanagida, T. and Tsujimoto, Y. (2000) Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c. J. Biol. Chem. **275**, 12321–12325

21 Tsujimoto, Y. and Shimizu, S. (2000) VDAC regulation by the Bcl-2 family of proteins. Cell Death Differ. **7**, 1174–1181

22 Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y. and Tsujimoto, Y. (2001) Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. J. Cell. Biol. **152**, 237–250

23 Tsujimoto, Y. and Shimizu, S. (2002) The voltage-dependent anion channel: an essential player in apoptosis. Biochimie **84**, 187–193

24 Vander Heiden, M. G., Chandel, N. S., Schumacker, P. T. and Thompson, C. B. (1999) Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell **3**, 159–167

25 Vander Heiden, M. G., Chandel, N. S., Li, X. X., Schumacker, P. T., Colombini, M. and Thompson, C. B. (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. Proc. Natl. Acad. Sci. U.S.A. **97**, 4666–4671

26 Vander Heiden, M. G., Li, X. X., Gottleib, E., Hill, R. B., Thompson, C. B. and Colombini, M. (2001) Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. J. Biol. Chem. **276**, 19414–19419

27 Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A. and Martinou, J. C. (1998) Bax-induced cytochrome *c* release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. J. Cell Biol. **143**, 217–224

28 von Ahsen, O., Renken, C., Perkins, G., Kluck, R. M., Bossy-Wetzel, E. and Newmeyer, D. D. (2000) Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. J. Cell Biol. **150**, 1027–1036

29 Waterhouse, N. J., Goldstein, J. C., von Ahsen, O., Schuler, M., Newmeyer, D. D. and Green, D. R. (2001) Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. J. Cell Biol. **153**, 319–328

30 Uren, A. G., O'Rourke, K., Aravind, L. A., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. and Dixit, V. M. (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol. Cell **6**, 961–967

31 Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S. and Frohlich, K. U. (2002) A caspase-related protease regulates apoptosis in yeast. Mol. Cell **9**, 911–917

32 Ligr, M., Madeo, F., Frohlich, E., Hilt, W., Frohlich, K. U. and Wolf, D. H. (1998) Mammalian Bax triggers apoptotic changes in yeast. FEBS Lett. **438**, 61–65

33 Zha, H., Fisk, H. A., Yaffe, M. P., Mahajan, N., Herman, B. and Reed, J. C. (1996) Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol. Cell. Biol. **16**, 6494–6508

34 Priault, M., Camougrand, N., Chaudhuri, B. and Manon, S. (1999) Role of the C-terminal domain of Bax and Bcl-XL in their localization and function in yeast cells. FEBS Lett. **443**, 225–228

35 Manon, S., Chaudhuri, B. and Guerin, M. (1997) Release of cytochrome c and decrease of cytochrome *c* oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett. **415**, 29–32

36 Gross, A., Pilcher, K., Blachly-Dyson, E., Basso, E., Jockel, J., Bassik, M. C., Korsmeyer, S. J. and Forte, M. (2000) Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X(L). Mol. Cell. Biol. **20**, 3125–3136

37 Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y. and Reed, J. C. (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat. Cell. Biol. **2**, 318–325

38 Greenhalf, W., Stephan, C. and Chaudhuri, B. (1996) Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in *Saccharomyces cerevisiae*. FEBS Lett. **380**, 169–175

39 Minn, A. J., Kettlun, C. S., Liang, H., Kelekar, A., Vander Heiden, M. G., Chang, B. S., Fesik, S. W., Fill, M. and Thompson, C. B. (1999) Bcl-xL regulates apoptosis by heterodimerization-dependent and -independent mechanisms. EMBO J. **18**, 632–643

40 Belli, G., Gari, E., Aldea, M. and Herrero, E. (1998) Functional analysis of yeast essential genes using a promoter-substitution cassette and the tetracycline-regulatable dual expression system. Yeast **14**, 1127–1138

41 Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19–27

42 Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene **110**, 119–122

43 Wach, A., Brachat, A., Pohlmann, R., Philippsen, P., Sikorski, R. S., Michaud, W. A., Tugendreich, S. and Hieter, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10**, 1793–1808

44 Yaffe, M. P. and Schatz, G. (1984) Two nuclear mutations that block mitochondrial protein import in yeast. Proc. Natl. Acad. Sci. U.S.A. **81**, 4819–4823

45 Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M. and Forte, M. (1997) Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. Mol. Cell. Biol. **17**, 5727–5738

46 Zinser, E. and Daum, G. (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. Yeast **11**, 493–536

47 Ronicke, V., Graulich, W., Mumberg, D., Muller, R. and Funk, M. (1997) Use of conditional promoters for expression of heterologous proteins in *Saccharomyces cerevisiae*. Methods Enzymol. **283**, 313–322

48 Priault, M., Chaudhuri, B., Clow, A., Camougrand, N. and Manon, S. (1999) Investigation of bax-induced release of cytochrome c from yeast mitochondria permeability of mitochondrial membranes, role of VDAC and ATP requirement. Eur. J. Biochem. **260**, 684–691

49 Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J. and Manon, S. (1999) Comparison of the effects of bax-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome *c*. FEBS Lett. **456**, 232–238

50 Zha, H. and Reed, J. C. (1997) Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J. Biol. Chem. **272**, 31482–31488

51 Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C. and Kroemer, G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science **281**, 2027–2031

52 Kissova, I., Polcic, P., Kempna, P., Zeman, I., Sabova, L. and Kolarov, J. (2000) 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. FEBS Lett. **471**, 113–118

53 Pavlov, E. V., Priault, M., Pietkiewicz, D., Cheng, E. H., Antonsson, B., Manon, S., Korsmeyer, S. J., Mannella, C. A. and Kinnally, K. W. (2001) A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. J. Cell Biol. **155**, 725–731

54 Egan, B., Beilharz, T., George, R., Isenmann, S., Gratzer, S., Wattenberg, B. and Lithgow, T. (1999) Targeting of tail-anchored proteins to yeast mitochondria *in vivo*. FEBS Lett. **451**, 243–248
55 Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E. and Pfanner, N. (2001) Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. J. Cell Biol. **152**, 289–300
56 Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R. and Newmeyer, D. D. (2002) Bid, bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell **111**, 331–342
57 Lee, A. C., Xu, X., Blachly-Dyson, E., Forte, M. and Colombini, M. (1998) The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. J. Membr. Biol. **161**, 173–181

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58 Harris, M. H., Vander Heiden, M. G., Kron, S. J. and Thompson, C. B. (2000) Role of oxidative phosphorylation in Bax toxicity. Mol. Cell. Biol. **20**, 3590–3596
59 Gross, A., Jockel, J., Wei, M. C. and Korsmeyer, S. J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J. **17**, 3878–3885
60 Antonsson, B., Montessuit, S., Sanchez, B. and Martinou, J. C. (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J. Biol. Chem. **276**, 11615–11623
61 Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J. C. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome *c* release from mitochondria. Biochem. J. **345**, 271–278
62 Nechushtan, A., Smith, C. L., Lamensdorf, I., Yoon, S. H. and Youle, R. J. (2001) Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. J. Cell Biol. **153**, 1265–1276