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BH3-only protein Bim inhibits activity of antiapoptotic members of Bcl-2 family when expressed in yeast

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ABSTRACT

Proteins of the Bcl-2 family regulate programmed cell death in mammals by promoting the release of cytochrome *c* from mitochondria in response to various proapoptotic stimuli. The mechanism by which BH3-only members of the family activate multidomain proapoptotic proteins Bax and Bak to form a pore in mitochondrial membranes remains under dispute. We report that cell death promoting activity of BH3-only protein Bim can be reconstituted in yeast when both Bax and antiapoptotic protein Bcl-X$_L$ are present, suggesting that Bim likely activates Bax indirectly by inhibiting antiapoptotic proteins.

1. Introduction

In mammalian cells, two major apoptotic pathways – the extrinsic and the intrinsic – are activated in response to either external or internal apoptotic stimuli, respectively. The proteins of the Bcl-2 family are key regulators of intrinsic (also known as mitochondrial) pathway [1]. They integrate diverse pro-death signals and translate them into the single universal death-inducing event – the release of cytochrome *c* from mitochondria, which is considered a commitment point of the pathway [2]. The family consist of both proapoptotic proteins, which promote the release of cytochrome *c* and antiapoptotic proteins that inhibit the release of cytochrome *c* from mitochondria. All family members share a sequence homology in up to four homologous regions – Bcl-2 homology domains (BH1-BH4). Based on their activity and presence of BH domains Bcl-2 proteins can be divided into three subfamilies. While multi-domain proapoptotic members of the family – Bax and Bak, which contain three BH domains (BH1-BH3), are the actual executioners of cytochrome *c* release as they participate at

the creation of a pore in outer mitochondrial membrane, their activity is regulated by other two subfamilies. Antiapoptotic subfamily of proteins containing all four BH domains, such as Bcl-X$_L$ and Bcl-2, inhibit their pore-formation activity and proapoptotic members referred to as 'BH3-only' subfamily, since they contain only BH3 domain, stimulate their activity in response to diverse proapoptotic stimuli [3].

Bim is one of the BH3-only proteins involved in the apoptosis induced by stimuli, such as antigen receptor ligation, Ca^{2+} flux, UV irradiation or taxol treatment. In living cells, Bim is sequestered to cytoskeleton by binding to the light chain of the dynein [4] and upon apoptotic stimulus it is released from the complex and activates the formation of the pore in the mitochondrial membrane by Bax or Bak. The controversy remains on whether Bim and other BH3-only proteins induce formation of the pore by directly activating multi-domain proapoptotic proteins (Bax, Bak) or whether they rather inhibit the antiapoptotic proteins (e.g., Bcl-X$_L$, Bcl-2).

Several isoforms of Bim are produced by alternative splicing of the Bim transcript. The longest form Bim$_{EL}$ is produced from unspliced mRNA, while in other isoforms, e.g., Bim$_L$ and Bim$_S$, excision of introns results in internal deletions [5]. Isoforms of Bim differ in the pro-death potency with short isoform (Bim$_S$) being the most and extra long isoform (Bim$_{EL}$) being the least potent. The different potency of these proteins may be due to different regulation of

their activity resulting from absence of the domains of the protein that potentially interact with other proteins, for example the absence of the dynein binding domain in Bim_S.

Although yeast do not have homologues of Bcl-2 proteins, it's been shown that yeast *Saccharomyces cerevisiae* can be used as a simple model system, in which to study the action of both multidomain proapoptotic and antiapoptotic members in the Bcl-2 family following their ectopic expression. Expression of proapoptotic protein Bax in yeast results in permeabilization of mitochondrial membranes, release of cytochrome *c* and ultimately to cell death [6,7]. Coexpression of antiapoptotic protein Bcl-X_L, on the other hand, suppresses the action of Bax on mitochondrial membrane and rescues cells from Bax induced cell death [7]. In order to address the questions of mechanisms that underlie the action of Bim, we have extended this model system by introducing 'BH3-only' protein Bim.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The yeast strains used throughout this study are CML282 (*MATa ura3-1*, *ade2-1*, *leu2-3,112*, *his3-11,15*, *trp1-Δ2*, *can1-100*, *CMV$_p$* (*tetR-SSN6*)::*LEU2*) [8], CML282/GAL-BAX [9] and CML282/GAL-BAK [10]. Latter two strains contain coding sequence of murine Bax or Bak N-terminally tagged with HA epitope, downstream from GAL1/10 promoter, integrated into genomic *HIS3* locus in CML282 background. Versions of these strains with *POR1* gene deleted (CML282Δ*por1*, CML282Δ*por1*/GAL-BAX and CML282Δ*por1*/GAL-BAK) were generated as described earlier [9].

Cells were grown on synthetic complete media (SC) containing the indicated carbon sources and lacking appropriate amino acids. Yeast were transformed by standard lithium acetate protocols [11].

To express Bim_{EL}, sequences encoding murine Bim were amplified by PCR using 5′-AGTCGGATCCATGGCCAAGCAACCTTCTGA-3′ and 5′-CTGAGAATTCAAGCTTGATCCTGTCAATGCCTTCTC-3′ primers, cleaved with *Bam*HI and *Eco*RI and subcloned into YCp-type vector p416MET25 downstream of the *MET25* promoter [12]. From resulting plasmid (p416MET-BIMEL), analogous plasmid containing Bim_S (p416MET-BIMS) was prepared by PCR (5′ phosphorylated 5′-GCTTCCATACGACAGTCTCAG-3′ and 5′-TTGCGGTTCTGT CTGTAGGGA-3′ as primers) and ligation of ends of generated product.

To assess the growth potential of individual strains, cells were grown overnight in 2% glucose containing media, diluted to $A_{600} = 0.5$ and 10 µl of serial 5-fold dilutions were spotted on test plates. Growth was assessed following incubation at 28 °C for 3 days.

To assess growth of yeast in liquid culture a procedure described in Weber et al. [19] was followed. Briefly, cells grown in SC (2% glucose) were resuspended in SC containing 2% raffinose and 0.1% galactose and no methionine to induce expression of Bax and Bim, diluted to $A_{600} = 0.1$ and cultivated at 28 °C. A_{600} was measured at indicated time points. To assess the viability, at corresponding times, aliquots containing 500 cells were plated on solid YPD (1% yeast extract, 2% peptone, 2% glucose) media. Colonies were counted after 3 days at 28 °C.

2.2. Preparation of protein extracts, immunoblotting and cell fractionation

Protein extracts were prepared by alkaline lysis and trichloroacetic acid (TCA) precipitation [13], separated by electrophoresis on 12.5% SDS–polyacrylamide gels and transferred to nitrocellulose membrane by electroblotting. Antibodies used for protein detection were a mouse monoclonal directed against the HA epitope (F-7, Santa Cruz Biotechnology), OxPhos Complex IV subunit II (COXII, Molecular Probes), phosphoglycerate kinase (Molecular Probes) and rabbit polyclonal against Bim/BOK (Chemicon International). Immunoblots were incubated with appropriate HRP-conjugated secondary antibodies (Promega) and binding visualized using a chemiluminescent substrate (Pierce).

Mitochondria were prepared as described previously [14,15]. Briefly, cells were converted to spheroplasts by enzymatic digestion with Zymolyase 20T (Seikagaku Kogyo) and homogenized with a glass-teflon homogenizer. The homogenate was centrifuged at $3000 \times g$, and the resulting supernatant centrifuged at $10\,000 \times g$ to pellet heavy membranes enriched in mitochondria. The supernatant from this centrifugation was recentrifuged at $10\,000 \times g$ and subsequent supernatant containing the cytosol loaded on to SDS–polyacrylamide gels. The heavy membrane fraction was washed with homogenization buffer prior to separation on SDS–PAGE.

For alkali treatment, the mitochondrial pellet was resuspended at 0.25 mg protein/ml in freshly prepared 0.1 M sodium carbonate, pH 11.5, and incubated on ice for 30 min. Membranes were pelleted by centrifugation at $150\,000 \times g$ while proteins removed from mitochondria by alkali treatment were precipitated from the supernatant by addition of TCA to 7%, centrifuged, and washed with acetone. Both alkali-extracted proteins and membrane fractions were analyzed by SDS–PAGE and Western blotting.

3. Results and discussion

In mammalian cells, Bim and other BH3-only proteins sense the state of the cell and trigger Bax and Bak to induce the release of cytochrome *c* from mitochondria upon various signals. The mechanism of the activation of Bax and Bak by Bim, however, remains under dispute. Some data indicate that at least some of the BH3-only proteins, including Bim, may induce oligomerization of Bax and a translocation of Bax to the mitochondria directly [16,17]. This would involve the physical binding of the BH3-only protein to the inactive cytosolic Bax, inducing a conformational change that would enable not only mitochondrial translocation of Bax but also oligomerization of Bax once inserted into mitochondrial membranes or Bak. In contrast, other data indicate that BH3-only proteins can bind antiapoptotic Bcl-2 family members, such as Bcl-X_L and Bcl-2, inhibiting their ability to counter the actions of proapoptotic family members like Bax and Bak, resulting in the activation of these proapoptotic molecules indirectly [18].

In order to investigate the mechanisms involved in proapoptotic action of BH3-only protein Bim, we have expressed the murine Bim in yeast *S. cerevisiae*. First we have transformed common yeast strain, CML282, with plasmids containing coding sequence of murine Bim gene downstream of *MET25* promoter and assessed the viability of resulting strain expressing Bim. As shown in Fig. 1b, protein of appropriate molecular weight corresponding to Bim_{EL} isoform was expressed. Expression of Bim did not have any effect on viability of the yeast strain expressing protein as compared to the strain transformed with the control plasmid (Fig. 1a) and no other evident phenotype has been observed in this strain. Since in mammalian cells Bim regulates apoptosis by modulating activity of other Bcl-2 family members, we have tested the ability of Bim to modulate the death- or survival-promoting activity of Bax and Bcl-X_L, respectively. To find out whether Bim modulates activity of Bax directly we have assessed the viability of yeast strains expressing both Bim and Bax. As described earlier [9], amount of Bax protein expressed in these strains is proportional to the concentration of galactose in media. In yeast strains expressing Bax, viability was dramatically compromised on the media with concentration of
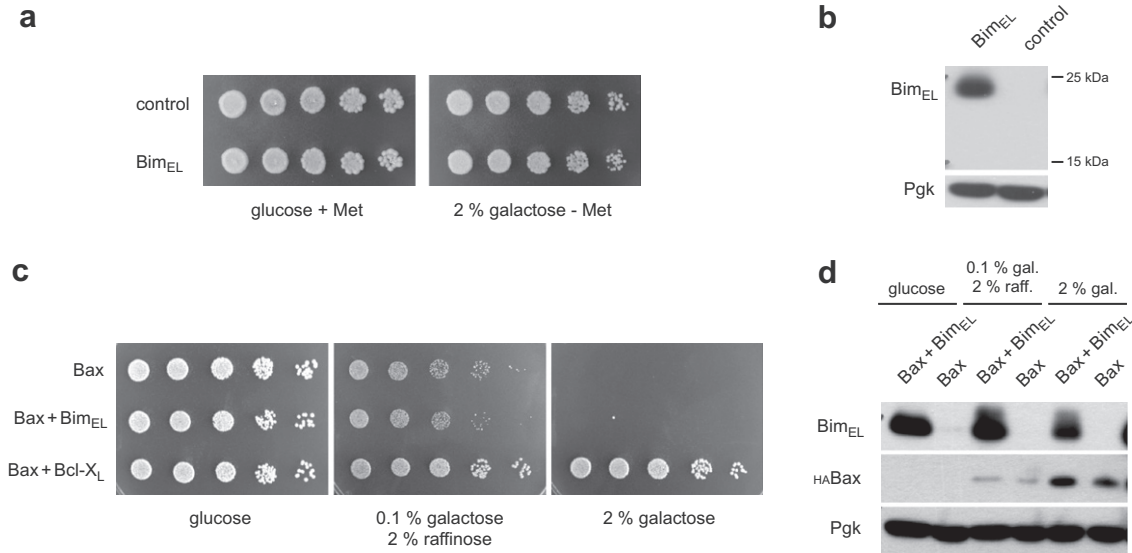
Fig. 1. Expression of Bim has no effect on growth of wild type and Bax-expressing yeast cells. (a) CML282 cells transformed with p416MET-BimEL (Bim$_{EL}$) or empty vector (control) were cultivated in glucose media and cell suspensions were spotted onto SC plates containing indicated carbon source with or without methionine to repress or to derepress the MET-BimEL, respectively. Growth was assessed after 3 days. (b) Expression of Bim$_{EL}$ and phosphoglycerate kinase (Pgk, a loading control) was detected by immunoblotting. (c) CML282/GAL-BAX cells transformed with p416MET-BimEL (Bax+Bim$_{EL}$) or empty vector (Bax) were cultivated in glucose media and cell suspensions were spotted on SC plates containing indicated carbon source. Medium was supplemented with 2 μg/ml doxycycline to support viability of control strain CML282-BAX-BCLXL (Bax+Bcl-X$_L$). Growth was assessed after 3 days. (d) Expression of Bim$_{EL}$, Bax and phosphoglycerate kinase (Pgk, a loading control) under relevant cultivation conditions was detected by immunoblotting.
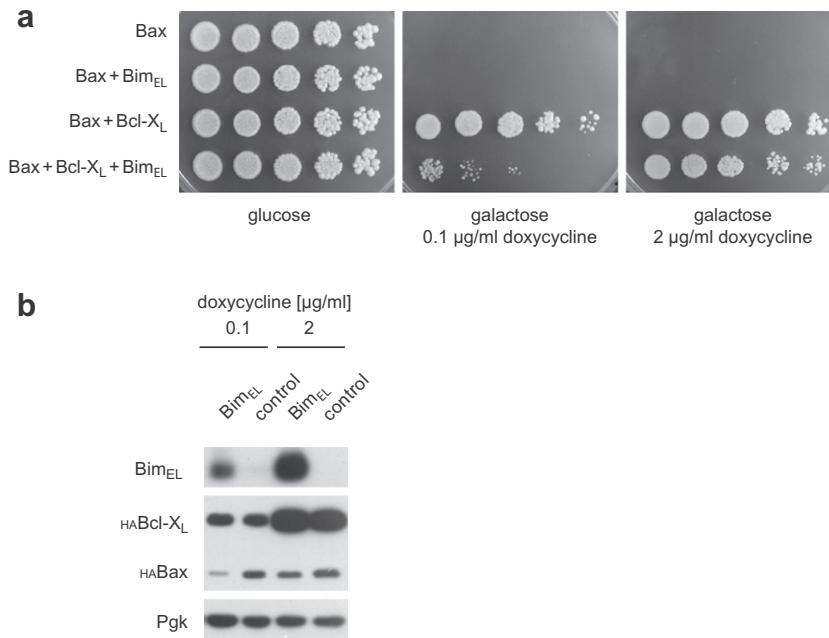

Fig. 2. Expression of Bim inhibits pro-survival activity of Bcl-X$_L$. (a) CML282/GAL-BAX cells transformed with TET-BCL-XL plasmid together with either p416MET-BimEL or empty vector were cultivated in glucose media and cell suspensions were spotted onto SC plates containing indicated carbon source and concentration of doxycycline. Growth was assessed after 3 days. (b) Expression of Bim$_{EL}$, Bax, Bcl-X$_L$ and phosphoglycerate kinase (Pgk, a loading control) under relevant cultivation conditions was detected by immunoblotting.

galactose higher than 0.1%. Coexpression of Bim did not noticeably increase the sensitivity of yeast towards Bax mediated killing, as shown by unchanged viability of strains expressing Bax and Bim compared to strains expressing Bax alone, on any concentration of galactose, especially in the range close to 0.1% (Fig. 1c), indicating that in this experimental setting Bim does not potentiate the action of Bax by direct interaction.

To test whether Bim is able to inhibit antiapoptotic activity of Bcl-X$_L$, we have tested the effect of expression of Bim on cell

survival of yeast strains coexpressing Bax and Bcl-X$_L$. The viability of strains expressing Bax and Bcl-X$_L$ either together with Bim or without Bim was assessed by spotting serial dilutions of cells to plates with 2% galactose and various increasing concentration of doxycycline. Strains, in which no Bim was expressed, maintained growth on plates with concentration of doxycycline of 0.1 μg/ml and higher due to sufficient expression of antiapoptotic Bcl-X$_L$ [9]. Decrease of viability of strains expressing Bim was observed as compared to strains without Bim (Fig. 2). Effect of Bim became
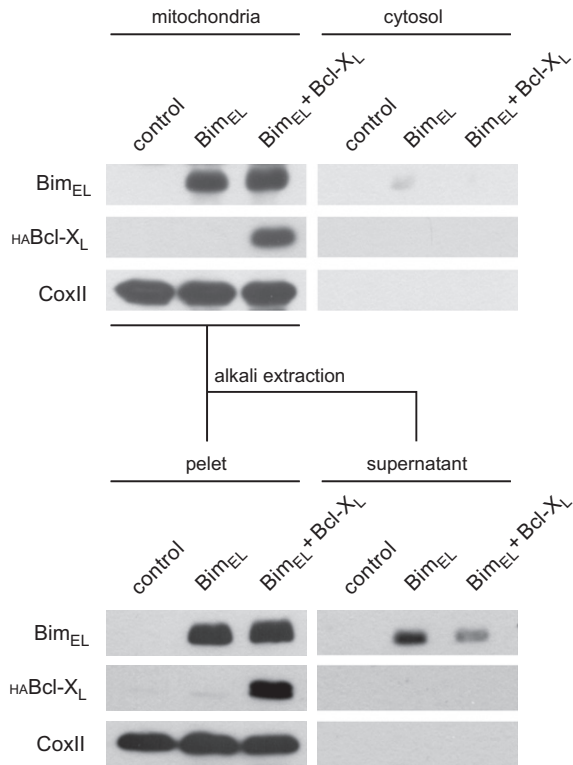
Fig. 3. Bim_{EL} is integrated into mitochondrial membrane. CML282 cells harboring p416MET-BimEL, TET-BCL-XL plasmids or respective empty vectors were grown in SC (0.25% glucose) without methionine (to support the expression of Bim_{EL}) supplemented with 2 µg/ml doxycycline (to support the expression of Bcl-X_L), converted to spheroplasts, homogenized and separated into soluble cytosolic and heavy-membrane fractions enriched in mitochondria by differential centrifugation. Fifty micrograms of protein of each fraction were separated by SDS–PAGE. Mitochondrial fractions were 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 Bim_{EL}, HA (HA-Bcl-X_L) and CoxII (inner mitochondrial membrane integral protein).

less apparent with increasing concentration of doxycycline and thus increasing amount of Bcl-X_L protein expressed. Essentially same results were obtained with yeast strain coexpressing proapoptotic protein Bak instead of Bax and antiapoptotic Bcl-2 instead of Bcl-X_L (Supplementary Fig. 1).

As these findings seemed to contradict the observation that expression of Bim (short isoform Bim_S) interferes with growth of yeast strain expressing Bax in the absence of antiapoptotic protein (e.g., Bcl-X_L or Bcl-2) when growth is assessed in liquid culture [19], we tested the growth of strain coexpressing Bim_{EL} and Bax, as well as strain coexpressing Bim_S together with Bax, in the same experimental setting as described in Weber et al. [19]. In this experiment, we have not observed any significant effect of expression of either Bim_{EL} or Bim_S on the growth of cell culture when

these were coexpressed with Bax (Supplementary Fig. 2a). At each time point we have also tested the viability of the cultures. Cultures coexpressing either isoform of Bim with Bax consisted of equal portion of viable cells as strain expressing Bax alone (Supplementary Fig. 2b), indicating that coexpression of Bim and Bax in this setting does not affect neither the growth rate nor the viability of yeast.

Results described above demonstrated that when murine Bim together with Bax and Bcl-X_L were expressed in yeast, Bim was able to inhibit antiapoptotic activity of Bcl-X_L while it did not potentiate the proapoptotic activity of Bax (or Bak) when no antiapoptotic proteins were present. While this favorizes indirect model of Bax activation by Bim over the direct model, the latter cannot be disproved completely as Bax expressed in yeast exhibits a cell killing activity even when no other Bcl-2 family members are present, indicating that it is at least partially active already.

To examine cellular localization of Bim, the cytosolic fraction and heavy-membrane fraction enriched in mitochondria were isolated from yeast strains expressing either Bim or Bim together with Bcl-X_L. Western-blot analysis of these fractions showed that in both strains Bim was present in in mitochondria enriched fraction resembling the localization of mitochondrial marker CoxII (Fig. 3). The assessment of the integration of Bim into mitochondrial membranes by alkali treatment demonstrated that, independently on the presence of Bcl-X_L, Bim is integrated into membranes (Fig. 3), indicating that Bim contains intrinsic mitochondrial membrane localization signal that targets Bim to mitochondria in the absence of proteins that sequester Bim in cytosol in mammalian cells (e.g., dynein LC8) and that no interaction with Bcl-X_L is required for mitochondrial membrane targeting and integration.

Based on experiments with purified Bim_{EL} and isolated mitochondria it has been suggested that Bim induced permeabilization of mitochondrial membrane is dependent on voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane [20]. Although participation of VDAC has also been proposed for permeabilization of mitochondrial membranes by Bax and Bak, it has been established that the latter two proteins permeabilize membrane independently of VDAC [9,21] and that VDAC may participate on the permeabilization of mitochondria by another mechanism – opening of the mitochondrial permeability transition pore [22].

To test whether VDAC is required for the inhibitory effect of Bim on Bcl-X_L we have carried out the same set of experiments as those described above in the isogenic yeast strain with *POR1* gene encoding for VDAC deleted (Δpor1). In this strain, Bim had no effect on viability when expressed alone and did not influence the survival of the strain coexpressing Bax. As it did in a wild type yeast strain, the expression of Bim reduced the viability of the Δpor1 strain when Bcl-X_L and Bax were coexpressed (Fig. 4), clearly indicating that mitochondrial VDAC is not required for the Bim to induce cell killing.

Taken together, our experiments in yeast have shown that BH3-only protein Bim likely activates Bax and Bak indirectly by inhibiting
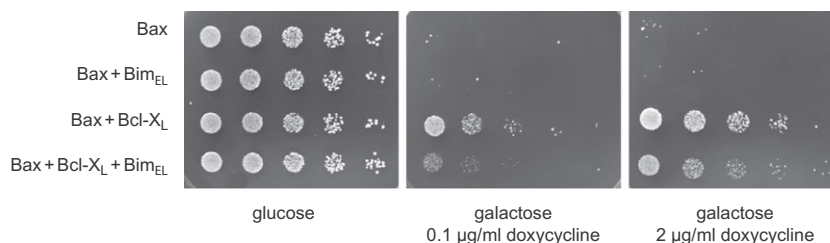
Fig. 4. Mitochondrial VDAC is not required for activity of Bim_{EL}. CML282/Δpor1/GAL-BAX harboring the TET-BCL-XL plasmid together with either pMET-BimEL plasmid or empty vector were cultivated in glucose media and cell suspensions were spotted onto plates containing indicated carbon source and concentration of doxycycline. Growth was assessed after 3 days.

antiapoptotic proteins Bcl-X$_L$ and Bcl-2, and that the action of Bim does not appear to be dependent on presence of VDAC in mitochondrial membrane.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.07.027.

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