Role of the C-terminal domain of Bax and Bcl-x_L in their localization and function in yeast cells

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Abstract **It has been suggested that the C-terminal domain of Bcl-2 family members may contain a signal anchor sequence that targets these proteins to the mitochondrial outer membrane. We have investigated the consequence of deleting this domain upon cytochrome *c* release in yeast strains that coexpress truncated forms of Bax (i.e. BaxΔ) and Bcl-x_L (i.e. Bcl-x_LΔ). We find that (i) BaxΔ is as efficient as full-length Bax in promoting cytochrome *c* release, but Bcl-x_LΔ has remarkably reduced rescuing ability compared to full-length Bcl-x_L; (ii) full-length Bcl-x_L protein acts by relocalizing Bax from the mitochondrial fraction to the soluble cytosolic fraction; (iii) Bax undergoes N-terminal cleavage when expressed in yeast, which is prevented by coexpression of Bcl-x_L, suggesting that Bcl-x_L may mask the cleavage site of Bax through a direct physical interaction of the two proteins.**

Key words: Apoptosis; Bax; Bcl-x_L; Cytochrome *c*; Yeast mitochondrion

1. Introduction

Apoptosis has been termed 'programmed cell death', which occurs under physiological circumstances. It aims to remove cells during development and regulation of tissue homeostasis in organs. Certain features of this program depend on the cell type, but some general characteristics are displayed by most mammalian cells. Whatever the method of induction of apoptosis, cytosolic signal transduction pathways are always recruited that end up in the nucleus where anti-apoptotic genes are switched off and pro-apoptotic genes are switched on. This confers the apoptotic morphology on the cells which can be monitored via nuclear fragmentation, chromatin condensation, cell shrinkage, membrane blebbing and budding of apoptotic bodies.

The Bcl-2 family of proteins has been identified as regulators of apoptosis, some being pro-apoptotic (Bax, Bad, Bcl-x_S) while others are anti-apoptotic (Bcl-2, Bcl-x_L). Members of this family have been identified through sequence comparisons with Bcl-2. Most family members contain four conserved domains (BH1–4; note that Bax does not contain the BH4 domain (Fig. 2)). Some of them also have a C-terminal hydrophobic α-helical domain that has been postulated to act as a membrane anchor (Fig. 2).

Two-hybrid and coimmunoprecipitation experiments have shown that Bcl-2 family members have the ability to homo- and heterodimerize. The BH3 domain is critical for these interactions but the C-terminal domain seems to be dispensable [1]. It has been proposed that the ratio between the pro- and the anti-apoptotic proteins rules the destiny of the cell. In addition, nuclear magnetic resonance and X-ray crystallographic analyses have highlighted some structural similarities to bacterial toxins and pore-forming proteins, suggesting that proteins of the Bcl-2 family may insert into membranes and act as ionic and/or protein channels [2,3]. Consistent with membrane localization, immunofluorescence data and immunohistostaining show that Bcl-2 family proteins are associated with the outer nuclear membrane, the endoplasmic reticulum and the outer mitochondrial membrane.

The possible pathways in which Bcl-2 family members act as regulators of apoptosis are still not fully elucidated. However, it is certain that specific mitochondrial events occur in response to induction of apoptosis. It has been observed that Bax expression triggers cytochrome *c* release from the mitochondrial intermembrane space to the cytosol [4]. The precise mechanism of this release is unknown. It has been proposed that the channel activity of the Bax protein is countered by a corresponding Bcl-x_L channel activity and this leads to a dysregulation of the ionic composition in the mitochondrial intermembrane space. The result is that the outer mitochondrial membrane is disrupted and thereby cytochrome *c* is released [5]. Another hypothesis envisages Bcl-2 as a negative regulator of a mitochondrial megachannel, the permeability transition pore (PTP). It is thought that heterodimerization with Bax nullifies Bcl-2 function and causes the opening of the PTP with subsequent mitochondrial swelling that leads to disruption of the outer mitochondrial membrane [6,7].

Although yeast cells do not express any Bcl-2-related proteins, it has been shown that at least one functional aspect of Bax and Bcl-x_L is conserved in yeast cells. Human Bax causes cytochrome *c* release and coexpression of Bcl-x_L suppresses this phenomenon [8]. In this communication, we have attempted to explore the role of the C-terminal domain in the Bcl-2 family of proteins, using yeast as a model system. The C-terminal sequence consists of a hydrophobic α-helix that should allow docking of proteins into host membranes. It is not clear from the published literature if the truncated forms of Bax and Bcl-x_L (i.e. BaxΔ and Bcl-x_LΔ, respectively) retain the same function and dimerization ability of the full-length molecules. Moreover, it is not known if the truncated molecules associate at all with the mitochondria.

In fact, contradictory results have been obtained for BaxΔ and Bcl-2Δ using similar cellular systems. On the one hand, Zha et al. [9] found that BaxΔ retains its toxicity and mitochondrial localization only in mammalian cells but not in yeast. The explanation was that in mammals, endogenous Bax in the outer mitochondrial membrane allows for interactions with BaxΔ, whereas this cannot happen in yeast since no endogenous Bax exists in this simple eukaryote. These results

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suggested that the targeting sequence in Bcl-2-like proteins may not be required if an endogenous membrane-bound protein allows for the interaction with an anchorless Bcl-2 homolog. However, when an endogenous regulator of cell death is absent, the membrane anchor of a pro-apoptotic protein would be essential for mitochondrial localization and imposition of cell death. In contrast to these findings, Nguyen et al. [10] have found that Bcl-2Δ is no longer targeted to the mitochondrial outer membrane in mammalian cells even in the presence of membrane-bound endogenous Bcl-2. But it must be said that the authors have not investigated the rescuing ability of the Bcl-2Δ protein [10].

Amidst these conflicting observations, we have tried to find out if the anchorless Bcl-2-like proteins are indeed targeted to the mitochondria or, in other words, if the C-terminal domain has any function in regulating cell death.

2. Materials and methods

2.1. Yeast strains

The strains used in this study are summarized in Table 1.

Human *bax* and *baxΔ172–192* genes were chemically synthesized with yeast codon bias, to encode α-isoform of both proteins. The genes were placed under the control of the *GAL10* promoter and fused to a fragment containing the *SUC2* transcription terminator with a 5′ DNA sequence encoding the c-*myc* epitope EQKLISEEDLNG [11]. These constructions were integrated at the *LEU2* locus of the wild type strain HT444.

The Bax and BaxΔ expressing strains thus obtained were transformed with a pDP83 plasmid (a CEN-14, YCp vector with *URA3* as yeast selection marker) that carries either *bcl-x_L* or *bcl-x_LΔ* human genes under the control of the *GAL10* promoter.

2.2. Cell fractionation

Cell culture conditions, spheroplast preparation and mitochondrion isolation have been fully described previously [8]. Besides, post-mitochondrial supernatants were centrifuged first at 130 000 × *g* for 35 min, which allows separation of the microsomal fraction (pellet) and cytosolic fraction (supernatant).

2.3. Cytochrome c detection

Cytochrome *c* release was measured by the spectrophotometric method on permeabilized spheroplasts, where the expression of Bcl-2 family proteins had been induced for 3 h by addition of 1% galactose in the culture medium.

Spectra were recorded in an Aminco DW2000 double-beam spectrophotometer, on dithionite-reduced versus H_2O_2-oxidized samples.

2.4. Western blotting

In each fraction, protein concentrations were estimated with the Lowry method. Samples were solubilized in 2% SDS at 65°C for 15 min, separated on 12.5% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes (Problott, Applied Biosystems). Membranes were blocked with 1% BSA in PBS/0.1% Tween 20 for 30 min, and subsequently blotted with primary monoclonal anti-c-*myc* antibodies (Santa Cruz Biotechnology) for 1 h. Then, the membranes were blotted with horseradish peroxidase-linked secondary antibodies (Immuno Pure, Pierce) for another hour. The protein bands were visualized with a chemiluminescence assay system (ECL, Amersham).

Table 1
Yeast strains

HT444	MATa, leu2, his4, ura3, lys2
Bax	MATa, his4, leu2, lys2, ura3, LEU2 = gal10p-*bax*
BaxΔ	MATa, his4, leu2, lys2, ura3, LEU2 = gal10p-*baxΔ*
Bax/Bcl-x_L	MATa, his4, leu2, lys2, ura3, LEU2 = gal10-*bax*, pDP83A/gal10p-*bcl-x_L* (URA3)
Bax/Bcl-x_LΔ	MATa, his4, leu2, lys2, ura3, LEU2 = gal10-*bax*, pDP83A/gal10p-*bcl-x_LΔ* (URA3)
BaxΔ/Bcl-x_LΔ	MATa, his4, leu2, lys2, ura3, LEU2 = gal10-*baxΔ*, pDP83A/gal10p-*bcl-x_LΔ* (URA3)
BaxΔ/Bcl-x_L	MATa, his4, leu2, lys2, ura3, LEU2 = gal10-*baxΔ*, pDP83A/gal10p-*bcl-x_L* (URA3)

3. Results and discussion

3.1. Cytochrome c release in strains expressing Bax or BaxΔ, and in strains that coexpress these proteins along with Bcl-x_L or Bcl-x_LΔ

Permeabilized spheroplasts were prepared from yeast strains, referred to as 'Bax' and 'BaxΔ' (i.e. strains that express full-length human Bax or the truncated Bax without its putative C-terminal transmembrane domain, in galactose-containing medium), to investigate if both forms of the pro-apoptotic protein are able to trigger cytochrome *c* release from the mitochondrial intermembrane space to the cytosol. Moreover, since it has been established that anti-apoptotic Bcl-x_L can reverse Bax effects in yeast as well as in mammalian cells, full-length and the truncated Bcl-x_L molecules (i.e. Bcl-x_LΔ that lacks the C-terminal transmembrane domain in Bcl-x_L) were coexpressed with Bax and BaxΔ in the 'Bax' and 'BaxΔ' strains. We were curious if Bcl-x_LΔ would be as efficient as full-length Bcl-x_L in rescuing cells from the effects of Bax and BaxΔ.

We confirmed that about 60% of total cytochrome *c* was released from mitochondria after a 3 h induction of Bax expression in galactose-containing medium (Fig. 1) [8]. Under identical experimental conditions, BaxΔ was as efficient as full-length Bax in promoting cytochrome *c* release (Fig. 1). These results differ from those reported by Zha et al. [9]. The differences may be due to the use of chimeric proteins. The constructs were made by fusing both Bax and BaxΔ to the LexA DNA-binding domain (DBD) or the B42 transactivation domain (TA). It is possible that the expressed BaxΔ fusion protein in particular [9] undergoes certain conformational changes so that the toxicity of BaxΔ in yeast is no longer manifested. Moreover, if the C-terminal truncation in Bax really abrogates mitochondrial targeting, as proposed by Zha et al. [9], a possible explanation for our BaxΔ-mediated cytochrome *c* release could be that the truncated molecule

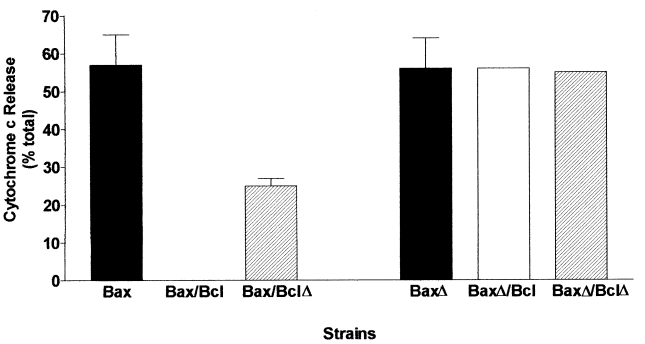

Fig. 1. Comparison of the ability of Bax and BaxΔ to promote cytochrome *c* release, and the efficiency of Bcl-x_L and Bcl-x_LΔ in preventing this. Cells were grown in YP liquid medium supplemented with 2% lactate, until late-exponential phase. Protein expression was induced for 3 h by adding 1% galactose. Spheroplasts were prepared and were permeabilized with nystatin as described in Section 2. After centrifugation, cytochrome *c* was measured in pellets and supernatants. Results present the percent of cytochrome *c* found in the supernatant.

triggers the event through a mechanism different from that used by full-length Bax which is found mostly in the outer mitochondrial membrane.

We have also confirmed that coexpression of full-length anti-apoptotic Bcl-x_L prevents cytochrome *c* release triggered by full-length Bax (Fig. 1) [8]. But we found that Bcl-$x_L\Delta$ only partially reverses the effects of Bax. Only about 25% of total cytochrome *c* is found in the supernatant of 'Bax/Bcl-$x_L\Delta$' cells (Fig. 1). From this observation, one can conclude that truncation of the putative C-terminal transmembrane domain partially impairs the protective function of Bcl-x_L. Additionally, we have observed that neither Bcl-x_L nor Bcl-$x_L\Delta$ was able to impede cytochrome *c* release triggered by truncated pro-apoptotic BaxΔ (Fig. 1). This raises a pertinent question regarding the true role of the C-terminal domain in interactions between members of the Bcl-2 family. Two-hybrid experiments indicate that the domain may be redundant for homo- or heterodimerization between members of the Bcl-2 family [1]. However, it is possible that the structural alterations induced by fusing genes with *lexA-DBD* and *B42-TA* domains may have concealed the real importance of the C-terminal domain. Our results seem to suggest that the C-terminal domain has a crucial role to play in the interactions between Bax and Bcl-x_L.

3.2. *Bax and Bax*Δ *localization*

Since our results relating to BaxΔ do not agree with previous findings, we decided to investigate if BaxΔ is found associated with the mitochondria after cell fractionation. We therefore isolated spheroplasts and mitochondria from Bax and BaxΔ strains after a 3-h expression, and separated the solubilized proteins by SDS-PAGE (Fig. 2). Expression of ~21 kDa Bax and ~18 kDa BaxΔ proteins was detected in lanes a and c, where proteins from whole spheroplasts were loaded. This indicates that both proteins are expressed

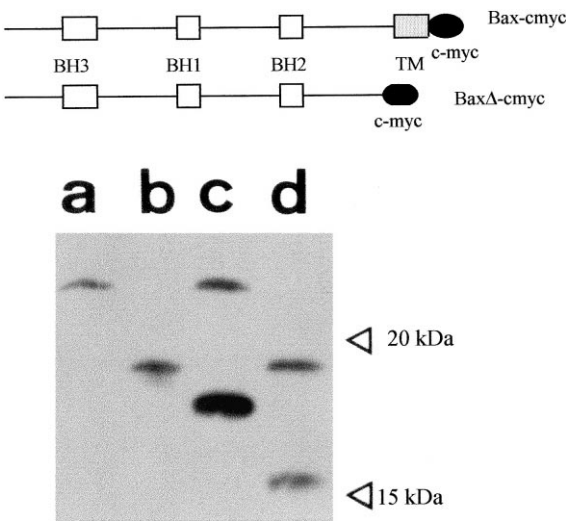

Fig. 2. Cleavage of Bax and BaxΔ. Cells were grown in YP liquid medium supplemented with 2% lactate, until late-exponential phase. Then Bax or BaxΔ expression was induced for 3 h by adding 1% galactose. For each fraction, 200 μg of proteins were separated on SDS-PAGE. Bax and BaxΔ were immunodetected as described in Section 2. Lane a, Bax in spheroplasts. Lane b, BaxΔ in spheroplasts. Lane c, Bax in mitochondrial fraction. Lane d, BaxΔ in mitochondrial fraction.

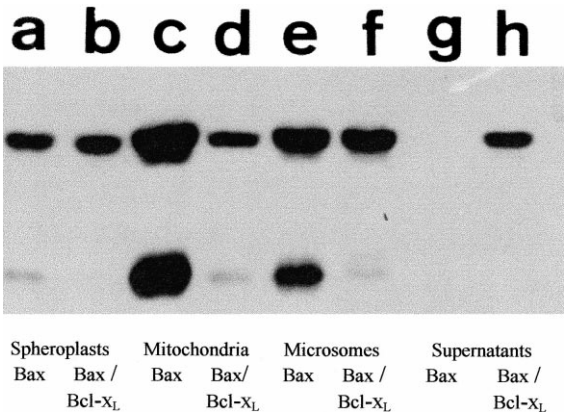

Fig. 3. Effects of Bcl-x_L on Bax localization. Cells were grown on YP medium supplemented with 2% lactate until 5 OD_{550nm} units. Then Bax and Bcl-x_L expression was induced for 3 h by adding 1% galactose. Proteins were measured by the Lowry method, and samples were analyzed by SDS-PAGE and Western blotting. Bax was immunodetected as described in Section 2. Lanes a and b, 150 μg of total protein. Lanes c, d, e, and f, 100 μg of total protein. Lanes g and h: 50 μg of total protein.

and that immunodetection is specific. These bands were also found in mitochondrial fractions (lanes b and d). Hence, it can be inferred that both Bax and BaxΔ are associated with mitochondria. This implies that the C-terminal domain of Bax is not necessary for targeting or for its docking into the outer mitochondrial membrane and it supports our earlier observation that Bax and BaxΔ are indistinguishable in their cytochrome *c*-releasing function.

Further analysis of the immunoblot reveals another unexpected phenomenon. Both Bax and BaxΔ appear to have undergone cleavage at their N-terminal ends, but only in the mitochondrial fractions. Cleavage of Bax and BaxΔ in mitochondria yields a ~17 kDa and a ~14 kDa protein, respectively (see lanes b and d, lower bands). Moreover, cleavage was also observed in microsomal fractions (Fig. 3, lane e). To the best of our knowledge, such a phenomenon has never been observed in mammalian cells, so we can draw no conclusions about its true physiological relevance. However, it would be interesting to know if the mitochondrial cleavage products of Bax and BaxΔ are also able to promote release of cytochrome *c*. Further experiments will be necessary to elaborate on this point.

In order to obtain an insight into the mechanism by which Bcl-x_L prevents Bax-induced cytochrome *c* release, we investigated Bax localization after fractionation of cells that express only Bax or coexpress both Bax and Bcl-x_L. Full-length Bax was detected in spheroplasts (Fig. 3, lanes a and b), showing that Bcl-x_L does not alter the levels of Bax expression. Mitochondrial fractions and microsomes contained not only the ~21 kDa band, but also the ~17 kDa one. But total amounts of Bax (i.e. the full-length 21 kDa Bax plus the 17 kDa cleavage product) are different in the mitochondrial fractions of 'Bax' and 'Bax/Bcl-x_L' cells (Fig. 3, compare the summation of intensities of the two bands in lanes c and d). This may suggest that coexpression of Bcl-x_L decreases the levels of Bax targeted to the mitochondria. Indeed, Bax proteins that were no longer found in mitochondrial membrane fractions of 'Bax/Bcl-x_L' cells were detected in the supernatant (Fig. 3, lane h). Interestingly, the soluble form of Bax is not seen in the absence of Bcl-x_L (Fig. 3, lane g). All these data

seem to indicate that expression of Bcl-x_L alters the intracellular localization of Bax.

It should also be noticed that there are dramatic differences in the levels of the cleaved 17 kDa form of Bax in mitochondrial/microsomal fractions obtained from 'Bax/Bcl-x_L' cells and 'Bax' cells. In 'Bax/Bcl-x_L' cells, the cleaved form is negligible. Moreover, the 17 kDa cleaved product of Bax is not seen in supernatants of 'Bax' cells. Since it is assumed that Bax and Bcl-x_L heterodimerize to negate each other's effects, it could be speculated that Bcl-x_L interacts with Bax in such a way that its cleavage site remains hidden.

4. Conclusion

Targeting of proteins to mitochondria usually implies that an N-terminal positively charged α-helical signal sequence inserts into the outer mitochondrial membrane. This insertion is $\Delta\Psi$-independent, ATP-dependent and temperature-sensitive [12]. Previous reports have claimed that the C-terminal putative transmembrane domain of the Bcl-2 family of proteins (such as Bax or Bcl-2) can act as a signal anchor sequence. This is based on the assumption that the proteins localize to mitochondrial membranes. In this paper, we have demonstrated that, at least for Bax, deletion of its C-terminal domain does not impair its association with mitochondrial subcellular fractions, nor does it impede the release of cytochrome *c* from the mitochondria to the cytosol. Thus, BaxΔ retains the same toxicity as full-length Bax in yeast cells. However, results obtained from experiments that use Bcl-$x_L\Delta$ do not allow an analogous conclusion. This is because Bcl-$x_L\Delta$ loses most of its rescuing ability, suggesting that the C-terminal domain of Bcl-x_L is important for its protective function during Bax-induced cytochrome *c* release. Full-length Bcl-x_L which annuls this release can efficiently perform this function by relocalizing Bax to the cytosol. It has yet to be determined if Bcl-x_L establishes direct physical contacts with Bax. A strong argument in favor of this idea is our observation that Bax is no longer found to be cleaved at its N-terminal side when Bcl-x_L is coexpressed.

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