The substitution of the C-terminus of bax by that of bcl-xL does not affect its subcellular localization but abrogates its pro-apoptotic properties

Lisa Oliver[a], Muriel Priault[b], Karine Tremblais[a], Marie-Thérèse LeCabellec[a], Khaled Meflah[a], Stéphen Manon[b], Francois M. Vallette[a,*]

[a] *Unité INSERM 419, 9 Quai Moncousu, 44035 Nantes Cedex 01, France*
[b] *UPR CNRS 9026, 1 rue Camille Saint Saëns, 33077 Bordeaux Cedex, France*

Received 23 October 2000; revised 17 November 2000; accepted 21 November 2000

First published online 5 December 2000

Edited by Vladimir Skulachev

Abstract The interaction of the anti-apoptotic members of the Bcl-2 family with mitochondria, through their hydrophobic C-terminus, has been proposed to play a crucial role in the execution phase of apoptosis. We report here that a substitution of the C-terminal end of pro-apoptotic bax by that of anti-apoptotic bcl-xL (baxCxL) does not modify its association with mitochondria in human and rat cells or in *Saccharomyces cerevisiae*. In addition, while bax sensitizes these cells to apoptotic stimuli, the construct baxCxL does not affect the apoptotic response in transfected cells. These results suggest that the C-terminus of bax plays an important role in apoptosis independently of its membrane addressing/targeting mechanism.

Key words: Apoptosis; Bax; Mitochondrion; DEVDase

1. Introduction

Members of the Bcl-2 proto-oncogene family are central regulators of the apoptotic programme [1–4]. The Bcl-2 gene encodes for a protein of 26 kDa which associates mainly with the mitochondrial outer membrane [1–4]. The Bcl-2 family includes both pro- and anti-apoptotic proteins which share several homologous structural domains such as the Bcl-2 Homology domains BH1, BH2 and BH3, and a stretch of hydrophobic amino acid residues located at the C-terminus [1–4]. The interaction of the latter domain with biological membranes is supposed to be a determinant in the subcellular localization of these proteins [1–4]. The BH domains are involved in the homodimerization or the heterodimerization of members of the Bcl-2 protein family among themselves or with other proteins, a property which is supposed to regulate their apoptotic function [1–4]. It has also been reported that bcl-2 and bax could form ionic channels in artificial membranes [4,5]. It has thus been postulated that Bcl-2 family members could exert their functions either through protein/protein interactions when anchored in the mitochondria through their hydrophobic C-termini or as pore-like membrane-embedded protein [2].

Bax, a pro-apoptotic member of this family, is normally located in the cytosol of healthy cells and is targeted to mitochondria upon induction of apoptosis [6,7]. The role of the C-terminus of bax in its integration into membranes and its pro-apoptotic function is unclear. Contradictory results of the effect of the deletion of the C-terminus of bax have been reported, both in mammals and in *Saccharomyces cerevisiae* [8–11]. We have recently shown, using a cell-free system, that the substitution of the C-terminus of bax by the equivalent segment of bcl-xL results in an efficient, although incomplete, association of the fusion protein baxCxL with mitochondria [8]. In the present work, we have investigated the influence of this C-terminal substitution on mitochondrial targeting and in pro-apoptotic function after stable expression in human and rat cell-lines or in *S. cerevisiae*.

2. Materials and methods

2.1. Reagents

Unless specified, all reagents used in this study were from Sigma (St. Louis, MO, USA). Monoclonal bax antibody (clone 4F11) was from Immunotech (France) and polyclonal bcl-xL antibody (B26630) was from Transduction Laboratories (Pharmingen, France); antibodies were used at a concentration of 1 and 5 μg/ml, respectively. The mitochondria-selective probe Mitotracker-Green FM was obtained from Molecular Probes (Interchim, France) and fluorogenic peptide Ac-DEVD-AMC was from Bachem (France).

2.2. Plasmids and cell transfections

The bax mutants with C-terminus substituted by that of bcl-xL (baxCxL), or deleted (baxΔC) were obtained as previously described [8] and subcloned into pRcCMV plasmid (Clontech, France). Bax and baxCxL were also subcloned into yeast tet-*off*-driven expression vector pCM189 carrying an URA3 selection marker, as described in [12].

The rat glioma A15A5 and human leukemic K562 cell-lines were transfected with control pRcCMV vector, pRcCMV-bax or pRcCMV-baxCxL. Plasmid DNA (5 μg) was introduced into 10^6 A15A5 or K562 cells by electroporation (GenePulser, BioRad) using 200 V/cm and 250 μF, transfected cells were selected in a medium containing G418 (250 μg/ml). Cells overexpressing human baxα were analyzed by immunoblot using the bulk of transfected K562 cells or in three different clones in the case of A15A5 cells.

Parental yeast strain W303-1A (*mat*a, *ade1*, *his3*, *leu2*, *trp1*, *ura3*) was transformed as described in [12] with pCM189-based plasmids expressing Bax or BaxCxL. Transformations were controlled by polymerase chain reaction on NaOH-lyzed colonies.

2.3. Cell fractionation and confocal analysis

Transfected K562 or A15A5 (2×10^6) cells were cultured in the presence or in the absence of staurosporine (1 μM), paclitaxel (5 μg/ml) or 1 min UV-B treatment. At different times after the in-

*Corresponding author. Fax: (33)-24-008 40 82.
E-mail: fval@nantes.inserm.fr

PII: S0014-5793(00)02330-9

duction of apoptosis, the cells were collected and centrifuged at 800×*g* for 10 min at 4°C. The cell pellets were washed with ice-cold phosphate-buffered saline (PBS), then resuspended in v/v CEB (250 mM sucrose; 50 mM HEPES pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, 1 mM EGTA and 1 mM PMSF) as previously described [13]. Cells were allowed to swell for 30 min on ice. The cells were then homogenized with 30 strokes in a 2 ml glass Dounce homogenizer. The homogenates were centrifuged at 800×*g* for 10 min at 4°C and the resulting supernatants centrifuged a further 15 min at 13 000×*g* at 4°C to obtain the mitochondrial pellets (P13). These supernatants were then centrifuged at 100 000×*g* for 30 min in an airfuge (Beckman, USA) and the resulting cytosolic fraction was further referred to as the S100. Bax and bcl-xL were detected in mitochondrial pellets and S100 using standard immunoblot methods.

The transfected cells were used for confocal and apoptosis analysis. For confocal analysis, the transfected cells were incubated with 25 μg/ml Mitotracker-Green FM for 30 min at 37°C, washed with PBS then fixed with 4% paraformaldehyde–0.19% picric acid in PBS for 30 min at room temperature. After saturation with 3% bovine serum albumin (BSA) in PBS, the cells were incubated with monoclonal anti-bax for 1 h at room temperature. After extensive washing, the second antibody, anti-mouse IgG coupled to rhodamine was added overnight at 4°C. Images were collected on a Leica TCS NT microscope with a 100×1.3 NA Fluotar objective (Leica, France).

2.4. Quantification of apoptosis

2.4.1. Lactate dehydrogenase (LDH) activity. Cell death was assessed by measuring the LDH activity released into the medium by healthy or by apoptotic cells. The LDH activity was measured using a cytotox 96® assay from Promega (France) according to the manufacturer's instructions.

2.4.2. DEVDase. Apoptosis was determined by measuring the caspase-3-like activity after induction of apoptosis as previously described [8,13]. In whole cell experiments, cells were washed several times with PBS, plated in 96-well plates and the caspase-3 fluorogenic substrate (Ac-DEVD-AMC) was added in the presence of 0.01% Triton X-100.

2.5. Yeast experiments

Yeast cells were grown aerobically in a semi-synthetic YNB medium (0.67% yeast nitrogen base, 0.1% ammonium sulfate, 0.5% potassium phosphate, 0.2% Drop-mix) supplemented with 2% lactate as a carbon source, auxotrophic requirements and 1 μg/ml doxycycline to prevent the expression of bax-derived proteins. The induction was induced by washing the cells three times then resuspending in the same medium without doxycycline. Growth was followed by the optical density of the cultures at 550 nm.

The localization of bax-derived proteins was observed in cells grown as above, after a 24 h induction of protein synthesis. The mitochondrial fraction was purified from spheroplasts generated by zymoliase treatment of the cells as described in [11]. Proteins were separated by SDS–PAGE, transferred on ProBlott membranes and Western blotting was done with a primary anti-bax monoclonal antibody directed against a peptide corresponding to the position 150–165 of bax (Calbiochem, France) and a secondary anti-mouse/peroxidase antibody (Jackson laboratories) and revealed by enhanced chemiluminescence (Amersham).

3. Results

3.1. Expression of bax, baxΔC and baxCxL in healthy and in apoptotic K562 cells

The human leukemic K562 cells were stably transfected with wild-type human bax (bax-α), baxΔC (deletion of amino acid 172–193 of bax-α) or baxCxL (human baxΔC fused with the amino acids 213–234 of human bcl-xL). The sequences of both bax and bcl-xL C-termini are illustrated in Fig. 1A. Cells were transfected with untagged bax constructs as we have previously shown that the addition of a HA-tag enhanced the association of bax with mitochondria [8]. We used the bulk of transfected K562 cells and the efficiency of the transfection was estimated by immunoblotting of bax. Although K562 cells expressed high amounts of endogenous bax, bax transfected cells exhibited a significant increased expression (Fig. 1B). Cells were fractionated as described in Section 2 and the efficiency of the fractionation was determined using actin as a cytosolic marker and the subunit IV of cytochrome *c* oxidase as a mitochondrial marker (data not shown). In healthy cells, most of the bax was found in the cytosol and only a small proportion was associated with mitochondria. As shown in Fig. 1, the addition of the transmembrane segment of bcl-xL did not enhance bax subcellular distribution. This result suggested that, in contrast to that observed in the cell-free system, the addition of the C-terminal end of bcl-xL did not enhance the mitochondrial binding of bax-α. However, this marginal association of bax or baxCxL with mitochondria in K562 cells appeared to depend upon the overexpression of bax and the presence of a hydrophobic C-terminus since no association of bax with mitochondria was found in pRcCMV or baxΔC transfected cells (Fig. 1B). Quite interestingly, bcl-xL which is highly expressed in K562, was present in both cytosolic and mitochondrial fractions, a repartition also found by Hsu et al. [6] in thymocytes (Fig. 1B). The induction of apoptosis by a brief UV-B treatment (see Section 2) caused the translocation of almost all of the cytosolic bax and bcl-xL to mitochondria. It should be noted that the nature of the C-terminus of bax did not influence the efficiency of this translocation (Fig. 1C). These results suggested that the addition of

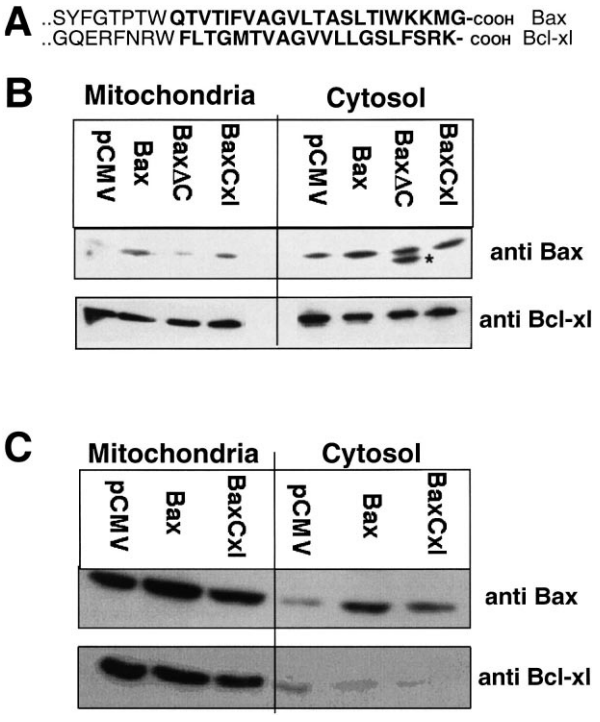

Fig. 1. Influence of the bcl-xL transmembrane segment on bax subcellular localization in the human leukemic K562 cell-line. A: Sequences of the C-terminus of human bax (amino acid 172–193) and bcl-xl (amino acid 213–234). B: Cell fractionation of control transfected K562 cells was performed as described in Section 2. P13 and S100 fractions were analyzed by immunoblot using antibodies raised against human bax. The same concentration of total S100 and P13 were used in order to visualize the actual cellular ratio. C: Similar analysis using apoptotic UV-B treated transfected cells.

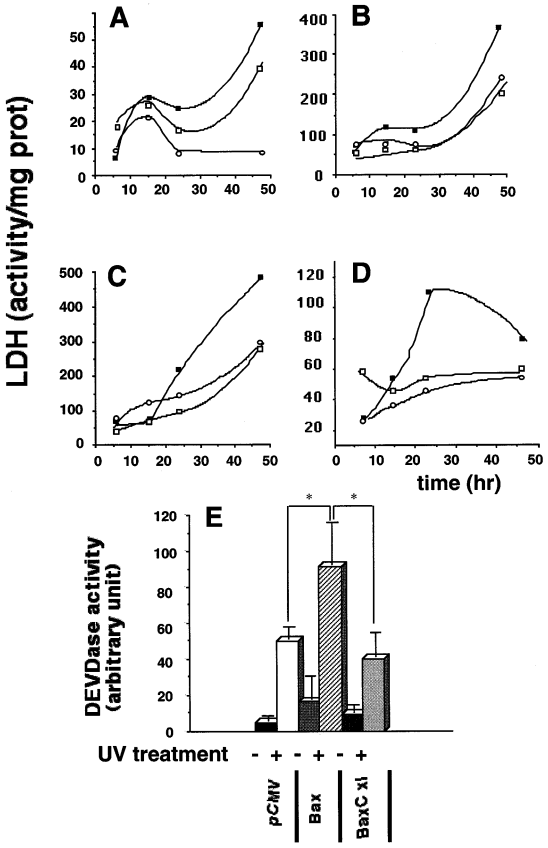

Fig. 2. Influence of the bcl-xL transmembrane segment on bax pro-apoptogenic activity in K562 cell-line. LDH activity release into the culture medium of healthy (A), paclitaxel- (B), staurosporine- (C) or UV-B-treated K562 cells (D) (cf. Section 2). bax (■), baxCxL (□) and pRcCMV (○) transfected cells. Experiments illustrated are representative of at least three different experiments. DEVDase activity in UV-B treated K562 cells, * indicated statistically significant different activities (E), data obtained from three independent experiments.

the C-terminal end of bcl-xL did not affect its subcellular localization in either healthy or apoptotic cells.

We induced apoptosis by several different stimuli (paclitaxel, UV or staurosporine) and cell death was assessed by the release of the cytosolic enzyme LDH into the culture media (Section 2). As illustrated in Fig. 2, the overexpression of bax sensitized K562 cells to all death stimuli while the presence of baxCxL had no effect on the rate or the extent of LDH release. However, in untreated cells, the extent of LDH-release remained elevated in both baxCxL and bax transfected cells compared to pRcCMV transfected cells (Fig. 2A). This result suggests a 'constitutive' toxicity of baxCxL similar to that observed with bax-α.

We quantified the level of apoptosis in these cells using the degradation of the fluorogenic peptide Ac-DEVD-AMC, a caspase-3 substrate, as previously described [8]. The results of the DEVDase assay were similar to that observed in the LDH assay, demonstrating a potentiation of apoptosis by bax. The difference between pRcCMV transfected cells and bax transfected cells was significant ($P = 0.0397$) but the difference between pRcCMV transfected cells and baxCxL was not significant ($P = 0.342$; Fig. 2E). It should be noted that no significant differences in the DEVDase activity were observed among untreated cells ($P = 0.42$).

3.2. BaxCxL does not sensitize A15A5 cells to apoptosis induced by different stimuli

In this study, we used three different clones of the rat A15A5 cell-line transfected with either pRcCMV, human bax (pRcCMV bax) or the baxCxL construct (pRcCMV-baxCxL). Bax and baxCxL transfected cells exhibited similar levels of bax protein as well as a similar distribution as determined by immunoblots using subcellular fractionation (data not shown) or by laser confocal microscopy (Fig. 3). In healthy cells, bax-α and baxCxL appeared to be mostly cytosolic with a preferential perinuclear localization (Fig. 3). Apoptosis was induced by different stimuli (UV-B treatment, staurosporine or paclitaxel) and cell death was determined by LDH release and by DEVDase activity. Induction of apoptosis induced a redistribution of bax-α and baxCxL to the mitochondrial membrane as previously shown in K562 cells (data not shown). We found that, as in K562 cells, transfection with bax-α sensitized A15A5 cells to all apoptotic stimuli with no differences between the response of pRcCMV and baxCxL transfected cells to apoptotic inducers, as illustrated in the case of cell death induced by a short UV-B treatment which was quantified using a DEVDase assay. As shown in Fig. 3G, the DEVDase activity was significantly increased in all transfected cells 24 h after the UV-B treatment. Nonetheless, a marked difference was observed between control (pRcCMV) and bax transfected cells as the DEVDase activity was two-fold higher in the latter cells ($P = 0.0481$). On the other hand, similar DEVDase activities were induced by the UV-B treatment in control pRcCMV and in baxCxL transfected A15A5 cells ($P = 0.645$). These results demonstrate that the ectopic expression of baxCxL does not potentiate apoptotic induction in A15A5 cells as observed previously in human cells.

3.3. Expression of bax and baxCxL in yeast

Yeast *S. cerevisiae* do not spontaneously undergo apoptosis and do not contain any analogs of the Bcl-2 family members, including bax. However, the heterologous expression of bax in yeast induced a cell death which was accompanied by typical characteristics of apoptosis such as cytochrome *c* release [14], DNA laddering and phosphatidylserine exposure [15]. This mortality and the accompanying phenotypes were prevented by the co-expression of bcl-2 or bcl-xL. When expressed in yeast, both bax and baxΔC have a mitochondrial localization and there was no significant difference between their lethal effect [11].

Bax and baxCxL were expressed under the control of the tet-*off* promoter. Under these conditions, the expression of bax induced a growth arrest of yeast cells (Fig. 4) followed by lethality, as already described [12]. In contrast, the expression of baxCxL did not induce any growth arrest of yeast cells (Fig. 4). Cell death as assayed by colony forming capacity after 24 h of induction of bax expression was 83% for bax transfected yeast and only 5% for baxCxL transfected cells.

The level of expression and the amount of both proteins in the mitochondrial fraction were identical (data not shown). Interestingly, the short form of bax, which appeared after a N-terminal cleavage accompanying mitochondrial translocation in yeast [11], was still present, and in even higher proportions, in baxCxL transfected cells (data not shown).

These results demonstrate that, as in rat and human cells, the substitution of the C-terminus of bax by the equivalent

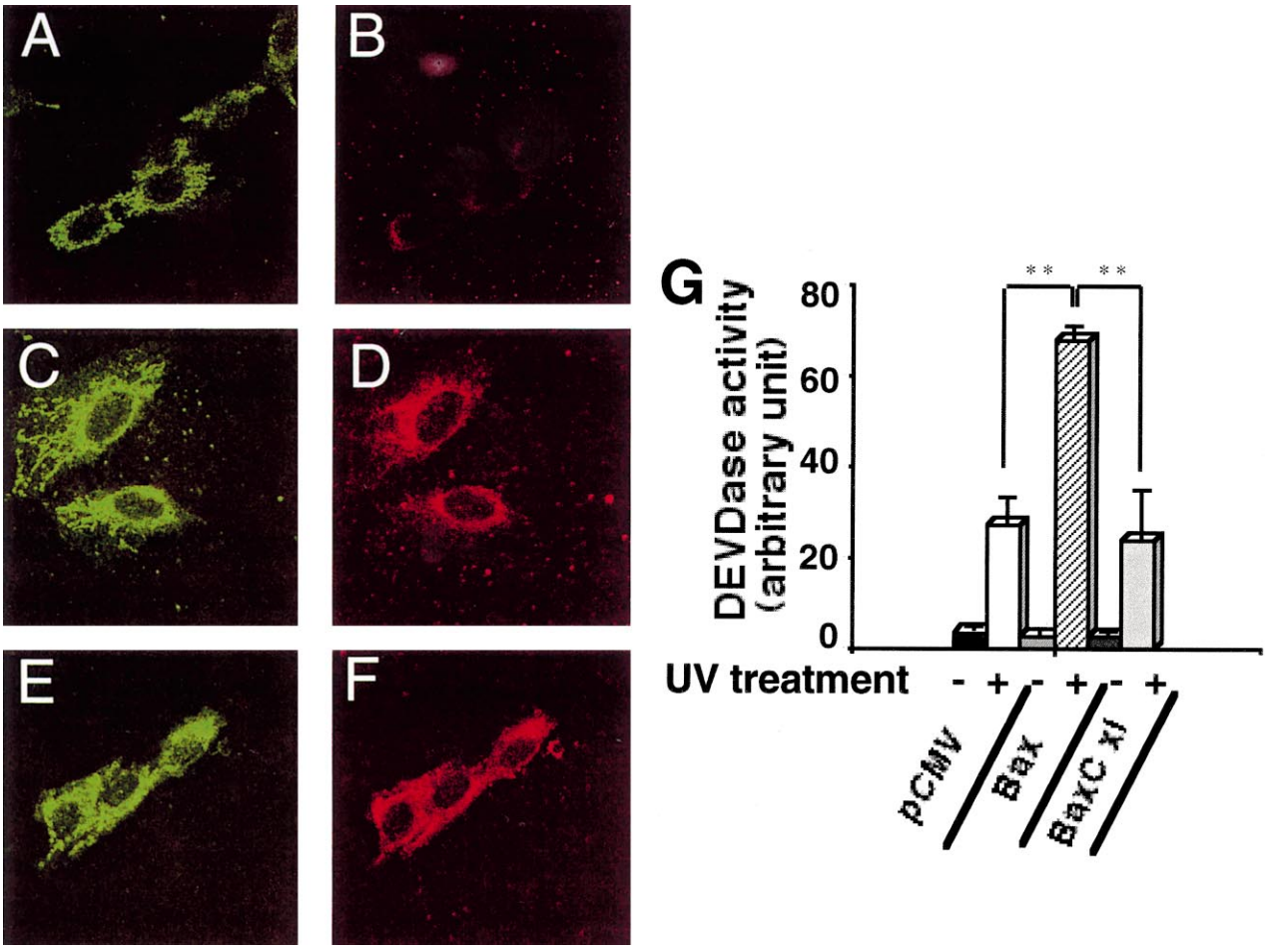

Fig. 3. Influence of the bcl-xL transmembrane segment on bax subcellular localization and pro-apoptogenic activity in the rat glioblastoma A15A5 cell-line. A–F: Confocal analysis of bax intracellular localization: double labelling with a fluorescent marker of mitochondria (Mitotracker, green) and bax (red) in A15A5 cells transfected with pRcCMV (A: mitotracker, B: bax); Bax (C: mitotracker, D: bax); BaxCxL (E: mitotracker, F: bax). Each image is representative of at least three different experiments. G: Induction of apoptosis in pRcCMV, bax or baxCxL transfected A15A5 cells. The DEVDase activity was measured, in three different clones for each plasmid, 24 h after the induction of apoptosis by a brief UV-B treatment (Section 2). ** indicated statistically significant different activities, data obtained from three independent experiments.

sequence of bcl-xL does not impair the mitochondrial localization of the protein but prevents its cytotoxic effect.

4. Discussion

Deletion of the C-terminal end of bcl-2 inhibits its anti-apoptotic function [16,17] and its substitution by that of cytochrome *b5* relocalizes bcl-2 to the endoplasmic reticulum which elicits its anti-apoptotic property towards some apoptotic stimuli [18]. In some cells, bax appears to be essentially cytosolic with only a small percentage of the protein associated with mitochondria [7]. The bulk of cytosolic bax is translocated to the mitochondria upon induction of apoptosis [6] and this translocation is one of the earliest rate-limiting steps in the executive phase of apoptosis [7]. Thus, the 'proper' mitochondrial localization of members of the Bcl-2 family is important in the control of apoptosis. The signals involved in bax addressing and incorporation into mitochondrial membranes are still unknown. It has been proposed that the C-terminus of bax is involved in its association with mitochondria, on the basis of its hydrophobic profile and by analogy with the equivalent segment in bcl-2 [1–4]. However, conflictual results on the importance of this segment in both bax pro-death function and intracellular localization in mammals and in yeast have recently been published [8–11]. Bcl-xL, like bcl-2, appears to be mostly mitochondrial [19] and this localization relies on its hydrophobic C-terminal [20]. Hsu et al. [6] reported that a significant part of bcl-xL is cytosolic in healthy cells and, like bax, translocates to mitochondria during apoptosis. We have recently observed that, in a cell-free system, the C-terminus of bax is not an addressing mitochondrial signal and that the substitution of this segment by that of bcl-xL leads to the binding of the chimeric bax protein (baxCxL) to mitochondria [8]. In this work, we used this construct as a putative constitutively mitochondria-bound bax. In the rat and human cellular models used in this study, this construct was preferentially not associated with mitochondria but, like wild-type bax-α and bcl-xL, remained partly cytosolic and was translocated to the mitochondria upon induction of apoptosis (Figs. 1 and 3). In yeast, bax-α and baxCxL have a similar mitochondrial localization (data not shown). Therefore, in the three cellular models, the substitution of the C-terminus of bax by that of bcl-xL does not affect the localization of the protein.

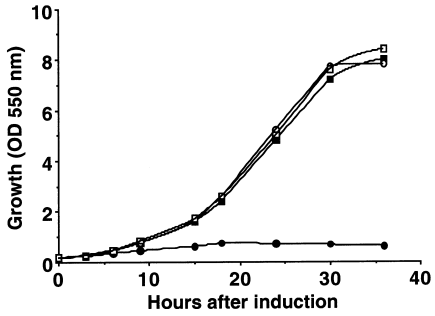

Fig. 4. Influence of the bcl-xL transmembrane segment on bax cytotoxicity and localization in *S. cerevisiae*. Cells were grown aerobically in YNB-lactate medium and induction of bax-α or baxCxL expression was induced by doxycycline-removal. A: Growth of yeast cells containing a plasmid expressing bax or baxCxL without or with induction of the proteins compared to that of yeast baxCxL. Doxocyclin induction: bax ●, baxCxL ○; absence of induction; bax ■, baxCxL □.

However, the substitution had a profound effect on bax-activity as it abolished its pro-apoptotic function in all cell types studied. Recently, Nechustan et al. [21] showed that the C-terminus of bax required a substitution at a strategic serine to become an addressing/anchoring signal. Interestingly depending on the nature of the amino acid substituted for the serine the mutation could provoke a gain or a loss of function [21]. Bax undergoes conformational changes that are required for its integration into mitochondrial membrane as a homodimer [21–23]. The N-terminus disengagement, identified by its recognition by specific antibodies, has been postulated to be an important step in bax activation in mitochondria [22]. It should be noted that in mammalian cells, once bound to mitochondria, we observed that baxCxL exposed its N-terminus as does wild-type bax (data not shown). In yeast, a N-terminal cleavage of bax occurs during mitochondrial translocation and this cleavage was also found with baxCxL, suggesting that the accessibility of baxCxL to the protease responsible for this cleavage is the same as for bax.

Another important putative feature in bax function is its interaction with the ATP/ADP translocator (ANT) of the mitochondrial inner membrane [24]. Interestingly, we observed an increase in the expression of ANT in bax transfected cells but not in baxCxL transfected cells (unpublished results). One could hypothesize that the presence of the C-terminus of bcl-xL hinders the capacity of bax to interact with ANT and thus its ability to induce apoptosis. In conclusion, our data support the idea that the C-terminus of bax could play an essential role in the control of its pro-apoptotic activity but not in its addressing to mitochondria.

Acknowledgements: This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Association pour la Recherche contre le Cancer and The Ligue Nationale contre le Cancer.

References

[1] Kroemer, G. (1997) Nat. Med. 3, 614–620.
[2] Reed, J.C. (1997) Nature 387, 773–776.
[3] Korsmeyer, S.J. (1999) Cancer Res. 59 (suppl), 1693s–1700s.
[4] Tsujimoto, Y. and Shimuzu, S. (2000) FEBS Lett. 466, 6–10.
[5] Schendel, S.L., Montal, M. and Reed, J.C. (1998) Cell Death Diff. 5, 372–380.
[6] Hsu, Y.-T., Wolter, K.G. and Youle, R.J. (1997) Proc. Natl. Acad. Sci. USA 94, 3668–3672.
[7] Gross, A., McDonnell, J.M. and Korsmeyer, S.J. (1999) Genes Dev. 13, 1899–1911.
[8] Tremblais, K., Oliver, L.J., Juin, P., LeCabellec, M.T., Meflah, K. and Vallette, F.M. (1999) Biochem. Biophys. Res. Commun. 260, 582–591.
[9] Zha, H., Fisk, H.A., Yaffe, M.P., Mahajan, N., Herman, B. and Reed, J.C. (1996) Mol. Cell Biol. 19, 6494–6507.
[10] Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997) J. Cell Biol. 139, 1281–1292.
[11] Priault, M., Camougrand, N., Chaudhuri, B. and Manon, S. (1999) FEBS Lett. 443, 225–228.
[12] Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J. and Manon, S. (1999) FEBS Lett. 456, 232–238.
[13] Juin, P., Pelletier, M., Oliver, L., Tremblais, K., Grégoire, M., Meflah, K. and Vallette, F.M. (1998) J. Biol. Chem. 273, 17559–17564.
[14] Manon, S., Chaudhuri, B. and Guérin, M. (1997) FEBS Lett. 415, 29–32.
[15] Ligr, M., Madeo, F., Fröhlich, E., Hilt, W., Fröhlich, K.U. and Wolf, D.H. (1998) FEBS Lett. 438, 61–65.
[16] Nguyen, M., Branton, P.E., Walton, P.A., Oltvai, Z.N., Korsmeyer, S.J. and Shore, G.C. (1994) J. Biol. Chem. 269, 16521–16524.
[17] Janiak, F., Leber, B. and Andrews, D.W. (1994) J. Biol. Chem. 269, 9842–9849.
[18] Zhu, W., Cowie, A., Wasfy, G., Penn, L., Leber, B. and Andrews, D. (1996) EMBO J. 15, 4130–4141.
[19] Gonzalez-Garcia, M., Perez-Ballestero, R.?, Ding, L., Duan, L., Boise, L.H., Thompson, C.B. and Nunez, G. (1994) Development 120, 33033–33042.
[20] Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D., Chang, B.S., Thompson, C.B., Wong, S.-L., Shi-Chung, N. and Fesik, S.W. (1996) Nature 381, 335–341.
[21] Nechustan, A., Smyth, C.L., Hsu, Y.T. and Youle, R.J. (1999) EMBO J. 18, 2330–2341.
[22] Goping, I.S., Gross, A./, Lavoie, N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J. and Shore, G.C. (1998) J. Cell Biol. 143, 207–215.
[23] Deshager, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antosson, B. and Martinou, J.C. (1999) J. Cell Biol. 144, 891–901.
[24] 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) Science 281, 2027–2031.