Involvement of the N-terminus of Bax in its intracellular localization and function

P.-F. Cartron, C. Moreau, L. Oliver, E. Mayat, K. Meflah, F.M. Vallette*

INSERM U419, IFR 26, 9 quai MONCOUSU, 44035 Nantes Cedex, France

Received 16 November 2001; revised 17 December 2001; accepted 19 December 2001

First published online 14 January 2002

Edited by Vladimir Skulachev

Abstract We have identified, using site-directed mutagenesis, a proline located at position 13 of Baxα (Bax) as crucial for the maintenance of its cytosolic conformation. The substitution of this proline by a valine results in a strong binding of Bax to mitochondria and to conformational changes monitored by a decreased sensitivity of Bax to mild proteolysis and the enhancement of its oligomerization state. Deletion of the C-terminus of Bax does not modify its intracellular localization. On the other hand, the pro-apoptotic activity of Bax is enhanced by a deletion of the C-terminus in the absence of the N-terminus but is decreased in its presence. These results suggest that both extremities functionally interact to control the activity but not the subcellular localization of Bax. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Bax; Conformation; Mutagenesis

1. Introduction

The control of apoptosis, a form of programmed cell death, is an essential feature for the maintenance of cellular homeostasis in healthy as well as in pathological tissue [1–3]. Proteins which belong to the Bcl-2 (B-cell lymphoma) family can either stimulate (Bax, Bak, Bad, Bid, etc.) or inhibit apoptosis (Bcl-2, Bcl-XL, Mcl-1, etc.) [4–6]. In viable cells, these proteins exhibit different subcellular localizations: Bcl-2 and Bak are mainly mitochondrial while BH_3 only pro-apoptotic proteins such as Bad and Bid are cytosolic [4–6]. On the other hand, Baxα (Bax) and Bcl-XL are both cytosolic and membrane-bound in non-apoptotic cells [7–11]. Upon the induction of apoptosis, Bax, Bid and Bad are targeted to the mitochondria and participate in the activation of the execution phase of apoptosis by triggering the release of mitochondrial apoptogenic proteins such as the cytochrome *c* [4–6]. The addressing and/or the insertion of these proteins in the mitochondrion is one of the rate-limiting steps in the activation of the mitochondrial pathway of cell death [7]. The anti-apoptotic proteins Bcl-2 and Bcl-XL are targeted and inserted into the mitochondrial outer membrane by their hydrophobic C-terminal extremities [12–14]. Since Bax displays a hydrophobic C-terminus, it has been proposed that this segment anchors this protein to the mitochondrial outer membrane [15]. However, the role of the COOH-terminus of human Bax (CT) in the membrane integration process and in its pro-apoptotic function is unclear as deletions, mutations or substitutions within this sequence have given contradictory results both in mammals and in *Saccharomyces cerevisiae* [10,16–21]. Goping et al. [17] showed that the NH_2-terminus of human Bax (NT) was involved in the regulation of Bax targeting to the mitochondria and thus called this sequence ART for apoptotic regulation of targeting. The deletion of ART caused a rapid association of Bax with the mitochondria and induction of cell death [17]. It has also be suggested that the cytosolic conformation of Bax is secured by the interaction of the hydrophobic CT with the NT [17,18,20]. In this work, we have examined by site-directed mutagenesis the amino acids involved in the ART function and the relationship between the NT and the CT of Bax.

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 was used at a concentration of 1 μg/ml in confocal microscopy experiments. The mitochondrion-selective probe Mitotracker Green FM was obtained from Molecular Probes (Interchim, France) and the fluorogenic peptide Ac-DEVD-AMC from Bachem (France).

2.2. Plasmids and cell transfections

N-terminal mutants of Bax were obtained by site-directed mutagenesis using the PCR-based Gateway™ method and were subcloned in pDEST plasmids according to the manufacturer's instructions (Invitrogen, Parsley, UK). Primers used for site-directed mutagenesis were Baxα (Bax) (5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGACGGGGAGCAGCCCAGAGGCGGG-3′), BaxΔART (5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGAAGACAGGGGCCCTTTTGCTTCAGTTCAT-3′), BaxΔS4L (5′- **GGGGACAAGTTTGTACAAAAAAGCAGGC**TTCATGGACGGGTTAGGGGAGCAGCCCAGAGGCGGG-3′), BaxS15L (5′-**GGGGACAAGTTT**GTACAAAAAAGCAGGCTTCATGGACGGGTCCGGGGAGCAGCCCAGAGGCGGGCCCACCCTCTCTGAAACAG-3′), BaxP8V (5′- **GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGGACGGGTCCGGGGAGCAGCTCAGAGGCGGG-3′), BaxP13V (5′-**GGGGACAAGTTTGTACAAAAAA**GCAGGCTTCATGGACGGGTCCGGGGAGCAGCCCAGAGGCGGCGGGGCCACCAGC-3′), BaxP8V/P13V (5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGACGGGTCCGGGGAGCAGCTCAGAGGCGGCGGGGCCACCAGC-3′), BaxP8L/R9G (5′-**GGGGACAAGTTTGTACAAAA**AAGCAGGCTTCATGGACGGGTCCGGGGAGCAGCTCGGAGGCGGG-3′), BaxΔC (5′-**GGGGACAAGTTTGTACA-**

*Corresponding author. Fax: (33)-24-008 4082.
E-mail address: fval@nantes.inserm.fr (F.M. Vallette).

Abbreviations: Bax, Baxα; BdGBM, Bax-deficient glioblastoma multiforme; doxo, doxorubicin; ART, apoptotic regulation of targeting; NT, NH_2-terminus of human Bax; CT, COOH-terminus of human Bax

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02227-5

AAAAAGCAGGCTACAAAAAAGCAGGCTATGCCACGTGGG-CGTCCCAAAGTAGGAGAGGAG-3′) and BaxC (5′-**GGGGAC-AAGTTTGTACAAAAAAGCAGGCT**TTCAGCCCATCTTCCAGA-TGGTGAGCGAGGCGGTGAGCA-3′).

A Bax-deficient cell line derived from a human glioblastoma multiforme (BdGBM) (Cartron et al., in preparation) was transfected with the empty vector (pDEST 12.2, Life Technologies) or Bax mutants cloned into this vector. Plasmid DNAs (5 μg) were introduced into 10^6 BdGBM cells by electroporation (GenePulser, Bio-Rad) using 200 V/cm and 250 μF; the transfected cells were selected in a medium containing neomycin (250 μg/ml) for 48 h. After selection, the bulk of transfected cells was used in in vitro experiments as previously described [10].

2.3. Acellular assay of Bax insertion

Mitochondria were prepared from normal rat liver and the cell-free association of Bax with the mitochondria was performed as described previously [21]. Briefly, ^{35}S-Met (Amersham, France) labeled proteins were synthesized from cDNAs using the TNT coupled transcription/translation system from Promega (France). Post-translational insertion of labeled proteins into mitochondrial membranes and alkaline treatment of mitochondrial bound proteins were performed in a standard import buffer as described previously [21]. Radiolabeled proteins bound to the mitochondria were recovered in the pellet after centrifugation of the incubation mixture for 10 min at 4°C at $8000\times g$. ^{35}S-Met-Bax associated with isolated mitochondria were analyzed in an SDS–PAGE and scanned with a phosphorimager (Molecular Dynamics, France). The amount of proteins present in the gel slab was subsequently quantified with IPLab gel program (Signal Analytics, Vienna, VA, USA).

The association of Bax with Bid was performed using purified His-tagged full-length Bid described by Eskes et al. [22]. In mitochondrial association experiments, radiolabeled Bax and cold His-Bid were incubated together for 30 min before their addition to isolated rat liver mitochondria and the analysis of the insertion of Bax into the membranes.

2.4. Quantification of apoptosis

BdGBM cells (2×10^6 cells), transfected with the different bax constructs, were cultured in the presence or in the absence of doxorubicin (doxo, 2 μg/ml) for 24 h before quantification of apoptosis by measurement of the LDH and DEVDase activities as previously described [10]. Briefly, the LDH activity was measured using a Cytotox96® assay from Promega (France) in the culture medium after the induction of apoptosis while the cells were washed several times with PBS, plated in 96-well plates and the caspase-3 fluorogenic substrate (Ac-DEVD-AMC), added in the presence of 0.01% Triton X-100.

2.5. Confocal analysis

For confocal analysis, the cells were incubated for 30 min with 5 μg/ml Mitotracker Green FM at 37°C then fixed with 1:1 methanol/acetone for 15 min at −20°C. After saturation with 3% BSA in PBS the cells were incubated with anti-Bax antibody 1 h at room temperature. After extensive washing, a second anti-mouse IgG coupled to rhodamine was added overnight at 4°C. Images were collected on a Leica TCS NT microscope with an 100× 1.3 NA Fluotar objective (Leica, France).

2.6. Protein binding experiments

The coding regions of Bax and BaxΔART were subcloned in pDEST17 plasmid in order to add a histidine tag at the N-terminus of the coding region. The His-tagged proteins were expressed in bacteria and purified according to the manufacturer's instructions. In vitro His protein binding assays were performed as followed: Bax, BaxΔART and BaxP8V/P13V were in vitro translated and labeled with 35(*S*)-methionine as described above. Equal amounts of each labeled protein (4 fmol) were then diluted with 250 μl of binding buffer (142 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5 mM DTT, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and a mixture of other protease inhibitors) and incubated with an equimolar concentration of the His-tag-BaxΔART or His-tag-Bax immobilized on Ni-Sepharose at 4°C for 2 h. The resins were then extensively washed with binding buffer, and the His protein binding complexes were eluted with buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM DTT, and 250 mM imidazole before the analysis by SDS–PAGE and fluorography.

3. Results

3.1. Association of Bax mutants with mitochondria

To explore the relative importance of the NT and the CT in the association of Bax with mitochondrial membranes, we have constructed mutants which lack either the NT (deletion of amino acids 1–19) and/or the CT (deletion of amino acids 172–193) (Fig. 1A). We analyzed the association of 5 fmol Bax or its mutants with purified rat liver mitochondria using a cell-free assay (cf. Section 2). The ratio between ^{35}S-Met-labeled proteins and mitochondria was chosen as it was below the mitochondrial saturation level for Bcl-2 [21]. As shown in Fig. 1B, the ^{35}S-Met-labeled Bax bound poorly to rat liver mitochondria whereas a truncation of the NT (BaxΔART) dramatically increased the membrane association ($P=0.0004$) as previously shown by Goping et al. [17]. We also studied the influence of a deletion of the CT (ΔC) on the acellular binding of Bax (Fig. 1B). We found that the deletion of the hydrophobic construct had no influence on the binding of Bax to the mitochondria ($P=0.688$) as we have previously reported [21] but slightly enhanced that of ΔARTΔC ($P=0.0451$) (Fig. 1B). Alkali treatment of the mitochondrial

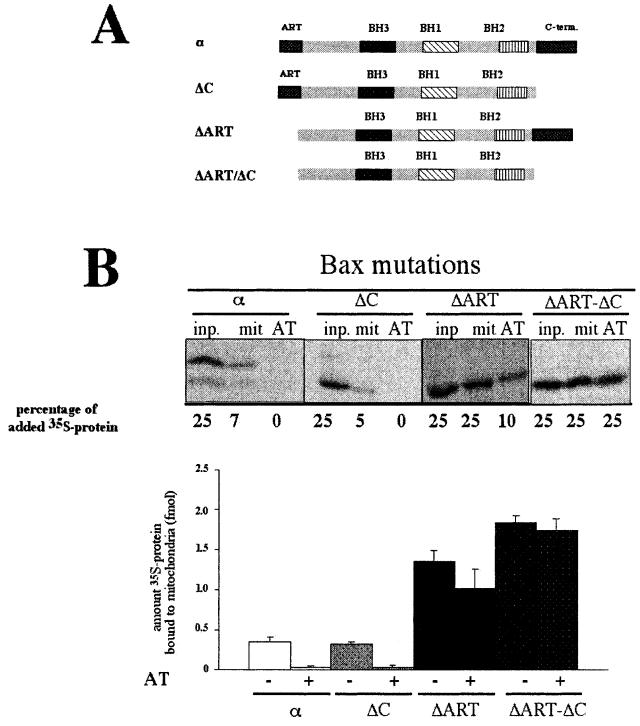

Fig. 1. Influence of the NT and the CT of Bax on its association with the mitochondria in a cell-free assay. A: Schematic representation of the ART sequence, CT and BH domains in Bax wild type and its mutants. B: Association of Bax mutants with mitochondria. ^{35}S-Met-labeled Bax mutants were incubated with isolated rat liver mitochondria (50 μg) in a standard import buffer as described in Section 2. At the end of the reaction (1 h at 37°C) the mitochondria were recovered by centrifugation and directly analyzed on SDS–PAGE and fluorography. Bax bound to the mitochondria (mit) or extracted with 0.1 M Na_2CO_3 (pH 11.5) (alkaline treatment: AT) as well as 25% of the initial input (total radiolabeled proteins initially added to mitochondria) (25% or 1 fmol) were analyzed in the same gel. Histograms show the amount of protein (fmol) present in the mitochondrial pellet before and after alkali treatment. Data shown are the mean ± S.D. obtained from at least four independent experiments.

bound Bax constructs indicated that only the ΔART constructs (ART and ΔARTΔC) were integrated into the membrane and/or strongly associated with an integral membrane protein (Fig. 1B). These results suggested that the NT and not the CT of Bax played a major role in the control of the targeting and/or the association of Bax to the membrane in this cell-free system.

A cell line derived from a human glioblastoma multiforme which did not express Bax (BdGBM, Cartron et al., in preparation) was transfected with the different mutants used in the cell-free assay. The bulk of transfected cells was used in this study to avoid bias due to the cloning procedure and also because similar levels of expression of *Bax* transgenes were found under these conditions (data not shown). Their subcellular localization was determined by laser confocal microscopy using Mitotracker Green to identify the mitochondria. Both Bax and BaxΔC transgenes were localized in the mitochondrial and in the cytosolic compartments. Deletion of the ART domain provoked a redistribution of Bax to the mitochondria, a tendency which was accentuated in the double deleted construct ΔARTΔC (data not shown). These results were qualitatively in agreement with the data obtained in the cell-free assay and thus underline the fundamental role of ART in the cytosolic retention of Bax.

3.2. A proline located in the N-terminal domain of Bax is essential for ART function

To address the question of the nature of the ART sequence involved in the retention of Bax in the cytosol of healthy cytosol, we engineered a series of mutations in the NT of Bax by site-directed mutagenesis as described in Section 2.2. As illustrated in Fig. 2A, the NT sequence of Bax contained several Pro which could play an important role in the control of the conformation. Indeed, the protein crystallography structure of Bax indicated that the first 12 amino acids in the NT were highly mobile, the second Pro being the 13th amino acid [23]. We have analyzed the effect of the substitution of these Pro as well as that of several Ser on the association of Bax with the mitochondria in the cell-free assay. The substitution of Ser-4 or Ser-15 by a Leu had little or no influence on the binding of Bax to the mitochondria (BaxS4L vs. Bax: $P = 0.09$; BaxS15L vs. Bax: $P = 0.336$) (Fig. 2B). On the other hand, the substitution of Pro-13 for a Val (P13V) enhanced significantly the association of Bax with mitochondria ($P = 0.003$), while the substitution of the other Pro (P8) present in the NT of Bax had no influence on the binding to the mitochondria ($P = 0.782$) (Fig. 2B). However, the combined substitution of both Pro had a more pronounced influence on Bax binding to mitochondria than that of Pro-13

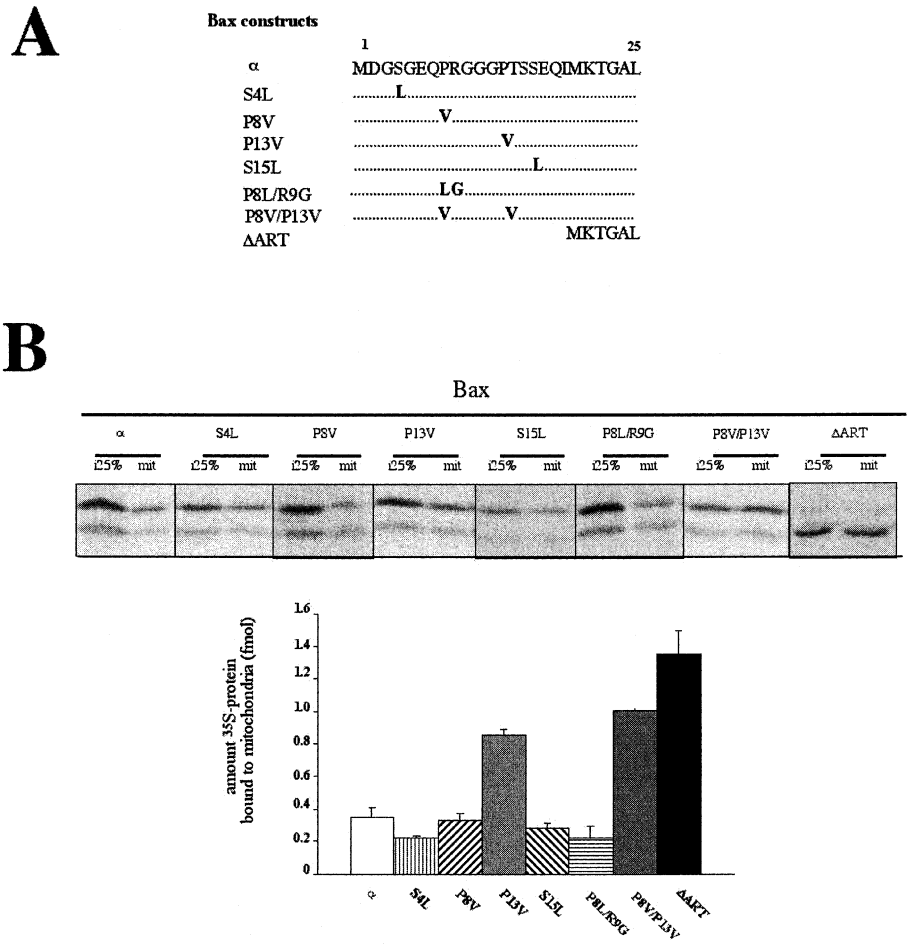

Fig. 2. Association of ART mutants with mitochondria in a cell-free assay and in vitro. A: Schematic representation of the different NT mutations used in the construction of BaxART mutant expression vectors. B: Association of BaxART mutants with purified rat liver mitochondria. The binding of BaxART mutants with isolated rat liver mitochondria was performed as described for Bax mutants in Fig. 1. Histograms show the amount of protein (fmol) present in the mitochondrial pellet. Data shown are the mean ± S.D. obtained from at least four independent experiments.

alone (Bax vs. BaxP8V/P13V: $P = 0.0017$; BaxP8V/P13V vs. BaxP13V: $P = 0.013$). Thus our results suggest a cooperation between the two Pro for the maintenance of the cytosolic conformation of human Bax. Interestingly, the sequence equivalent to PRG (amino acids 8–10) in human Bax is LGG in rat Bax [24], suggesting that the Pro-8 is not essential in the cytosolic retention of Bax. Indeed, the double mutation of the human Bax sequence for that of the rat had no influence on Bax binding to mitochondria (human Bax 'PRG' vs. rat Bax 'LGG': $P = 0.228$). Confocal analysis of Bax localization in BdGBM cells revealed that the substitution of Ser by a Leu had no effect on Bax localization while the substitution of one or two Pro by Val induced a mitochondrial redistribution of Bax (data not shown). The latter results suggested that the Pro located at position 13 of human Bax played an important role in the cytosolic conformation of human Bax and that the mutation of this amino acid caused the insertion of Bax into the mitochondria and/or its binding to an integral membrane protein.

3.3. Influence of the NT on Bax conformation and interaction with Bid

We and others have reported that a change in the Bax conformation could be monitored by its sensitivity toward mild proteolysis [18,21,25]. Thus, the influence of mutations in NT on its conformation was studied using sensitivity toward trypsin as described previously [21]. As shown in Fig. 3A, Bax was highly sensitive to mild proteolysis by trypsin while the P8V/P13V mutant showed some resistance toward trypsin. Similarly the ΔART construct was more resistant to proteolysis. Similar results were obtained with proteinase K (data not shown). On the other hand, deletion of the ΔC or the substitution of Ser-15 by a Leu had no effect on protease sensitivity. These results suggested that the specific modifications of the NT which enhanced Bax binding to mitochondria also altered its overall conformation.

Bax oligomerization has also been shown to be associated with its change in conformation at the onset of the apoptotic program [26,27]. His-tagged Bax and His-tagged BaxΔART purified proteins were incubated with in vitro labeled ^{35}S-Bax, ^{35}S-BaxΔART and ^{35}S-BaxP8V/P13V (see Section 2.6). As illustrated in Fig. 3B, no binding of BaxΔART with Bax was observed in this assay whereas both BaxΔART and BaxP8V/P13V bound vigorously to the His-tagged BaxΔART ($P = 0.0011$ and $P = 0.001$ respectively). On the other hand, no specific binding of ^{35}S-Bax, ^{35}S-BaxΔART and ^{35}S-BaxP8V/P13V was observed with His-tagged Bax (data not shown). The latter results suggested that the deletion or the mutation of NT enhanced the oligomerization of Bax.

Similarly, it has been reported that Bid induced a change in the conformation of Bax which in turn triggered its translocation to the mitochondria [22,27]. We co-incubated 5 fmol ^{35}S-Met-labeled Bax (i.e. 100 nM) with purified His-tagged full-length Bid at a concentration range from 1 to 100 nM. As reported earlier by Eskes et al. [22], the association of Bax with the mitochondria was increased when equimolar concentrations of Bid were used (data not shown and Fig. 3C). We

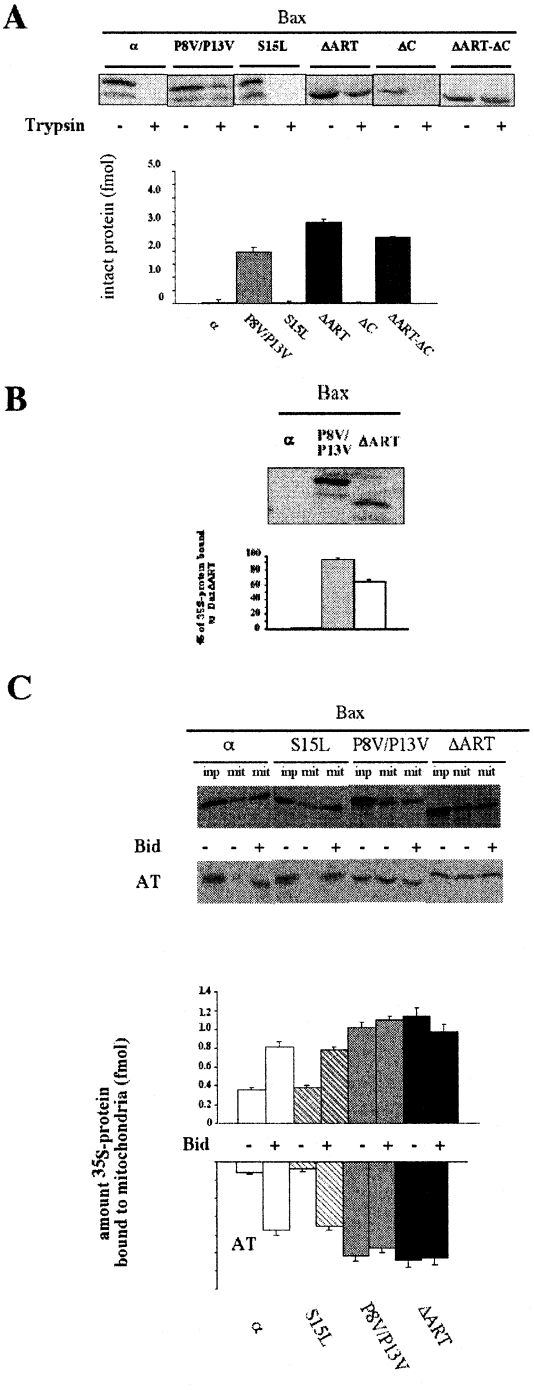

Fig. 3. ART mutations induce a change in conformation of Bax. A: Sensitivity of BaxART mutants to mild proteolysis. In vitro translated BaxART mutants were incubated with trypsin (1 mg/ml) for 15 min at 4°C. At the end of the incubation the proteolysis was stopped by the addition of 10 mg/ml soybean trypsin inhibitor and immediately heated in denaturating buffer before analysis on 15% SDS–PAGE. The data illustrated are representatives of at least three different experiments. B: Proteins binding assay. In vitro translated Bax, BaxΔART and BaxP8V/P13V were incubated with purified His-tagged BaxΔART as described in Section 2. The percentage of ^{35}S-labeled proteins bound to His-tagged ΔART was estimated after SDS–PAGE and fluorography using the initial input (4 fmol) as 100%. Data obtained from four different experiments. C: Influence of Bid on the acellular association of BaxART mutants with mitochondria. Bax, BaxS15L, BaxP8V/P13V and BaxΔART mutants were incubated in the absence or in the presence of 100 nM full-length Bid and bound radiolabeled proteins were recovered by centrifugation and treated as described in Fig. 1 (AT alkali treatment). Data presented are the mean ± S.D. obtained from at least three independent experiments.

incubated the NT mutants of Bax (BaxΔART, BaxS15L and BaxP13V) with 100 nM full-length His-tagged Bid and analyzed the association of the constructs with rat liver mitochondria. As illustrated in Fig. 3C, the mitochondrial association of BaxΔART and BaxΔP13V was not affected by a co-incubation with Bid while that of Bax and BaxS15L were significantly enhanced (Fig. 3). We found that constructs which associated with mitochondria in the presence of Bid such as Bax a or BaxS15L were membrane integrated as they were resistant to an alkaline extraction while BaxP13V, BaxP8V/P13V and BaxART membrane integration was not affected by the presence of Bid (Fig. 3C). To control the specificity of the Bid/Bax interaction, Bax was preincubated with purified His-tagged irrelevant proteins and in this case no increase in the binding of Bax was observed (data not shown). Thus, the Pro-13 substitution could bypass the Bid 'assistence' to bind mitochondria as reported for the ΔART mutant [28].

3.4. Effect of the NT mutations on Bax pro-apoptotic activity

We have analyzed the sensitivity to apoptosis of BdGBM cells transfected with the Bax mutants. It should be noted that the expression of the transgenes did not induce apoptosis in the absence of doxo, probably because of the extreme resistance of these cells to apoptosis (Cartron et al., in preparation). Apoptosis was induced with 2 μg/ml doxo and cell death was assayed by measuring the release of the cytosolic LDH into culture media and the cellular DEVDase activity as previously described [10]. The expression of *bax* transgene enhanced the sensitivity of BdGBM cells to doxo (Fig. 4). Quite interestingly, the deletion of the CT appeared to slightly inhibit the pro-apoptotic activity of Bax ($P = 0.004$) but enhanced that of BaxΔART ($P = 0.001$) (Fig. 4). As shown in Fig. 4, the DEVDase activity as well as the release of LDH were significantly increased in cells transfected with the Bax constructs mutated in the ART region which possessed a higher affinity for the mitochondria (e.g. ΔART, ΔARTΔC, BAXP13V and BAXP8V/P13V). On the other hand, no differences in the induction of apoptosis were observed between cells transfected with Bax and those transfected by the ART mutants S4L and S15L (Fig. 4).

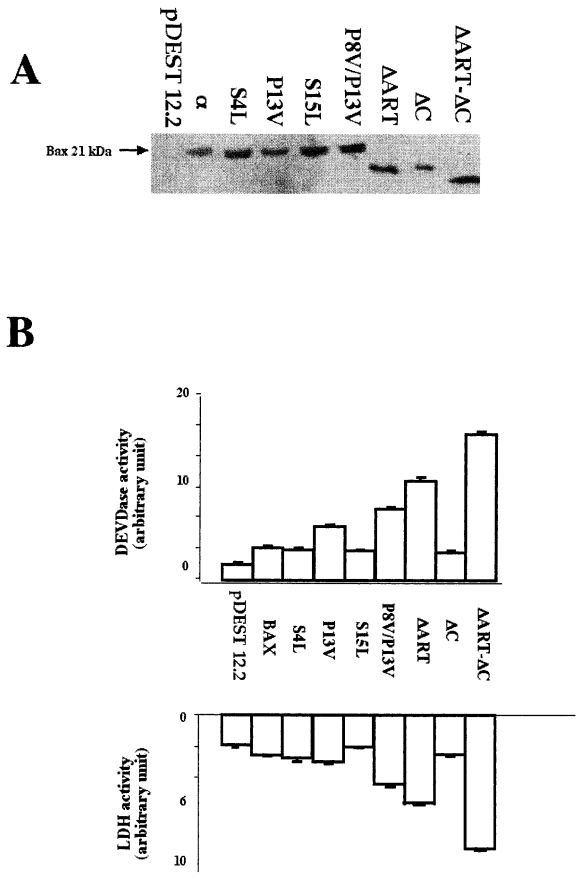

Fig. 4. Pro-apoptogenic activity of Bax mutants in vitro. A: Immunoblot analysis of the expression of bax transgenes in the BdGBM cells. B: The cellular DEVDase activity (top) and LDH activity release into the culture medium were assayed in doxo-treated BdGBM cells transfected with the different Bax mutants. Experiments illustrated are the mean ± S.D. of at least three different experiments.

4. Discussion

In the present work, we have investigated the nature of the amino acids involved in the cytosolic retention and/or mitochondrial sorting of Bax. We first addressed the question of the nature of the signal involved in Bax targeting to the mitochondria. By analogy with Bcl-2, the hydrophobic CT of Bax has been proposed to be the membrane addressing/insertion sequence. However, we and others have not observed a difference in the apoptotic subcellular localization of CT deleted Bax (BaxΔC) [16,19,21] while others found that this deletion impaired its targeting to mitochondria [8]. The complexity of the signal contained in the CT was emphasized by Nechustan et al. [20] who found that this sequence was not a membrane addressing signal unless a strategic Ser located at position 184 of human Bax was deleted or mutated into an Ala residue. In addition, these authors showed that the nature of the amino acid substituted could drastically affect the intracellular localization of the Bax mutants, as substitutions for a charged amino acid blocked the binding to mitochondria and even seemed to protect cells from cell death [20].

In this work, we show that the association of Bax constructs is not affected by the deletion of CT suggesting that this sequence is not a key determinant for Bax subcellular localization both in a cell-free system or in transfection experiments using a Bax-deficient cell line. On the other hand, the nature of the CT appears to affect its function as a substitution by the highly homologous CT of Bcl-XL does not affect its intracellular localization but inhibits the apoptotic action of Bax in mammals and in yeast [10]. In agreement with the latter results, we report in this work that the deletion of the CT increases Bax pro-apoptotic activity in the absence of ART (Fig. 4). It is likely that this inhibition results from the nature of the interaction of the CT with ART since the deletion of only the CT decreased the pro-apoptotic activity of Bax (Fig. 4). One could postulate that the removal of the CT favors a change in the conformation of the NT, however, insufficient to alter the cytosolic conformation of Bax imposed by the ART domain. Indeed, as noted by Nechustan et al. [20] a deletion of the CT exposed an otherwise hidden NT epitope recognized by the conformation-dependent monoclonal antibody 6A7. The recent resolution of the three-dimensional structure of Bax shows that the CT is located in the hydrophobic pocket occupied by the BH3 and, therefore, it has been proposed that it could inhibit the formation of homodimers of Bax [23], a prerequisite for the binding of Bax to mitochon-

dria. Alternatively, as recently suggested [10,29], the CT could regulate Bax activity by its interaction as a receptor binding domain with other mitochondrial proteins.

The cytosolic/mitochondria conformational transition of Bax seems to be controlled, at least partially, by its first 20 amino acids. Nechustan et al. [20] have shown that residues Pro-13–Ile-19 of the α1-helix are hidden in the cytosolic form of Bax and become accessible to antibody binding after the membrane insertion induced during apoptosis. Our results show that a substitution of the Pro-13 plays an essential role in the structure of this segment prior to the binding of Bax as a substitution of this Pro by a Val results in the dramatic relocalization of Bax both in a cell-free assay (Fig. 2) and in vitro (data not shown). Mutations of other amino acids located in the first 19 residues have little or no effect on the binding of Bax to the mitochondria (Fig. 2). The three-dimensional analysis of Bax revealed that the first 12 NT amino acids are highly flexible and thus are not likely to adopt an ordered conformation in solution [23,24]. However, as pointed out by Roucou and Martinou [30], the structure revealed by Susuki et al. [23] could represent only one of the possible folding states of Bax.

The interaction of Bax with Bid has recently been shown to be suppressed in BaxΔART [28] and we show in our work that the ART mutant P13V also bypasses the requirement of Bid for the mitochondrial integration of Bax (Fig. 3). NT deletion or mutation of Pro-13 appears to induce a change in conformation as judged by protease sensitivity, protein binding assay (Fig. 3) as well as the lack of interaction with Bid (Fig. 3). Khaled et al. [25] have shown a Bax mutant in which Asp-2 and Glu-6 are exchanged for Ala undergoes a change in the conformation viewed by an increased sensitivity to proteases. However, in the latter case the interaction of this mutant with the mitochondria was not documented [25]. The increase of the interaction between Bax mutants (Fig. 3C) suggests that the mutations of the Pro-13 or the removal of ART favor the dimerization among bax molecules. The latter process which is likely to occur through the BH3 domains could induce the association of these mutants to mitochondria [5]. The correlation between the property of NT mutants to dimerize, to bind mitochondria and their ability to induce apoptosis highlights thus the importance of the N-terminal end in the regulation of the structure/activity of Bax (Fig. 4). Recent results have shown that cleavage of Bax by calpain results in the generation of a very apoptogenic 18 kDa fragment [31]. It is noteworthy that in our condition, this cleavage is observed only at very late stages of apoptosis and after the integration of Bax into the membrane (data not shown) ruling out that this degradation is a prerequisite for the early activation of bax.

In conclusion, our results suggest that a proline present in the ART sequence is involved in the maintenance of an innocuous conformation of Bax which is only released at the onset of apoptosis by factors like BID. More importantly, the 'dialog' between NT and CT appears to be more critical for the control of bax activity than for its subcellular localization.

Acknowledgements: We thank Professor J.C. Martinou (University of Geneva) for providing us with the His-Bid construct. 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, The Ligue Nationale contre le Cancer. P.-F.C. was supported by a fellowship from the Ligue Départementale contre le Cancer Doubs/Montbeliard.

References

[1] Kerr, J., Wyllie, A. and Currie, A. (1972) Br. J. Cancer 26, 239–257.
[2] Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Cell 88, 347–354.
[3] Rudin, C.M. and Thompson, C.B. (1997) Annu. Rev. Med. 48, 267–281.
[4] Reed, J.C. (1997) Nature 387, 773–776.
[5] Gross, A., McDonnell, J.M. and Korsmeyer, S.J. (1999) Genes Dev. 13, 1899–1911.
[6] Desagher, S. and Martinou, J.C. (2000) Trends Cell Biol. 10, 369–377.
[7] Porter, A.G. (1999) Trends Cell Biol. 9, 394–401.
[8] 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.
[9] Hsu, Y.-T., Wolter, K.G. and Youle, R.J. (1997) Proc. Natl. Acad. Sci. USA 94, 3668–3672.
[10] Oliver, L., Priault, M., Tremblais, K., LeCabellec, M.T., Meflah, K., Manon, S. and Vallette, F.M. (2000) FEBS Lett. 487, 161–165.
[11] Martin, S., Toquet, C., Oliver, L., Cartron, P.F., Perrin, P., Meflah, K., Cuillère, P. and Vallette, F.M. (2001) J. Neuro-Oncol. 52, 129–139.
[12] Kutay, U., Hartmann, E. and Rapoport, T. (1993) Trends Cell Biol. 3, 73–75.
[13] Janiak, F., Leber, B. and Andrews, D.W. (1994) J. Biol. Chem. 269, 9842–9849.
[14] 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.
[15] Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Cell 74, 609–619.
[16] Zha, H., Fisk, H.A., Yaffe, M.P., Mahajan, N., Herman, B. and Reed, J.C. (1996) Mol. Cell. Biol. 19, 6494–6507.
[17] Goping, I.S., Gross, A., Lavoie, J.N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J. and Shore, G.C. (1998) J. Cell Biol. 143, 207–215.
[18] Gross, A., Jockel, J., Wei, M.C. and Korsmeyer, S.J. (1998) EMBO J. 17, 3878–3885.
[19] Priault, M., Camougrnad, N., Chaudhuri, B. and Manon, S. (1999) FEBS Lett. 443, 225–228.
[20] Nechustan, A., Smyth, C.L., Hsu, Y.T. and Youle, R.J. (1999) EMBO J. 18, 2330–2341.
[21] Tremblais, K., Oliver, L.J., Juin, P., LeCabellec, M.T., Meflah, K. and Vallette, F.M. (1999) Biochem. Biophys. Res. Commun. 260, 582–591.
[22] Eskes, R., Deshager, S., Antonsson, B. and Martinou, J.C. (2000) Mol. Cell. Biol. 20, 929–935.
[23] Suzuki, M., Youle, R.J. and Tjandra, N. (2000) Cell 103, 645–654.
[24] Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D. and White, E. (1996) Genes Dev. 10, 461–477.
[25] Khaled, A., Kim, K., Hofmeister, R., Mueggez, K. and Durum, S.K. (1999) Proc. Natl. Acad. Sci. USA 96, 14476–14481.
[26] Antonsson, B., Montessuit, S., Sanchez, B. and Martinou, J.C. (2001) J. Biol. Chem. 276, 11615–11623.
[27] Deshager, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, S., Antonsson, B. and Martinou, J.C. (1999) J. Cell Biol. 144, 891–901.
[28] Ruffolo, S.G., Nguyen, M., Goping, I.S., Gross, A., Korsmeyer, S.J., Li, H., Yuan, J. and Shore, G.C. (2000) Cell Death Diff. 7, 1101–1108.
[29] Lazebnik, Y. (2001) Curr. Biol. 11, R767–R768.
[30] Roucou, R. and Martinou, J.C. (2001) Cell Death Diff. 8, 875–877.
[31] Wood, D.E. and Newcomb, E.W. (2000) Exp. Cell Res. 256, 375–382.