Studies of the Interaction of Substituted Mutants of BAX with Yeast Mitochondria Reveal That the C-terminal Hydrophobic α-Helix Is a Second ART Sequence and Plays a Role in the Interaction with Anti-apoptotic BCL-xL*

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Hubert Arokium‡, Nadine Camougrand‡,François M. Vallette§, and Stéphane Manon‡¶

From the ‡UMR5095 CNRS/Université de Bordeaux 2, F-33077 Bordeaux cedex, France and §UMR601 INSERM/Université de Nantes, F-44035 Nantes cedex, France

The role of the two ends of the pro-apoptotic protein BAX in its interaction with mitochondria was challenged by assaying substituted mutants in yeast cells for the ability to bind and insert into the mitochondrial membrane and to promote the release of cytochrome c. Mutations at the N-terminal end confirmed the inhibitory function of this zone, known as apoptotic regulation of targeting (ART). On the other hand, mutations at the C-terminal end of the protein support the hypothesis that the hydrophobic helix α9 is not required for the insertion of BAX. In addition, three mutations (a T174D single substitution in the helix α9, a K189E/K190E double substitution at the end of the protein, and a P168A mutation in the loop before α9) exhibited a strong binding capacity, a strong insertion, as well as high ability to induce cytochrome c release. Considering the positions of these mutations and their potential effect on the movement of helix α9, we propose that the C-terminal end of the protein behaves like a second ART. Also, opposite to a mutation that changes the conformation of the N-terminal ART, the mutations in the C-terminal part of the protein impaired the inhibitory effect of anti-apoptotic BCL-xL over BAX insertion, suggesting that the conformation of the α9-helix plays a significant role in BAX/BCL-xL interaction.

Apoptosis, the main form of programmed cell death, is a highly regulated phenomenon. It is now largely accepted that mitochondria play a key role in regulating the apoptotic process by controlling the release of several proteins, termed apoptotic factors, required for the acquisition of apoptotic hallmarks (for review see Refs. 1 and 2). This release strictly depends on the action of a family of proteins, termed the BCL-2 family, that modulates the permeability of the outer mitochondrial membrane to the apoptotic factors (for review see Refs. 3 and 4). Moreover, the alteration of the bioenergetic capacity of mitochondria following the action of BCL-2 family members is thought to be further involved in the regulation of apoptosis by the production of reactive oxygen species (for review see Ref. 5) or the release of Ca2⁺ (for review see Ref. 6).

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† To whom correspondence should be addressed: UMR5095 CNRS/Université de Bordeaux 2, 1 Rue Camille Saint-Saëns, F-33077 Bordeaux, France. Tel.: 33-556-99-90-50; Fax: 33-556-99-90-51; E-mail: manon@ihgc.u-bordeaux2.fr.

Among the BCL-2 family members, the pro-apoptotic protein BAX plays a crucial role (for review see Ref. 7). BAX is expressed at basal levels in nearly all mammalian cells. In healthy cells, it behaves as a soluble protein and is localized in the cytosol; following apoptotic induction, the protein is translocated to the mitochondrial outer membrane where it behaves as a membrane-inserted protein (8, 9) and actively participates in the release of apoptotic factors. The molecular mechanisms governing the cytosol-to-mitochondria translocation of BAX therefore appear as a critical regulation in the implementation of apoptosis.

The changes in the physical properties of BAX accompanying its translocation strongly support the hypothesis that profound conformational changes are the basis of the change in the localization of the protein. Analysis of the primary structure of BAX reveals that, like all BCL-2 family members, it is a relatively hydrophilic protein with only a predicted hydrophobic α-helix of 18 residues close to the C-terminal end. The tertiary structure of the soluble form of BAX was determined by NMR (10). The protein is formed of nine α-helices, the last one being hydrophobic, and a poorly resolved 15-residue-long N-terminal end, having a high mobility. The role of this N-terminal zone has been revealed to be crucial in the regulation of addressing BAX to mitochondria; the deletion of this zone increases in vitro binding of the protein to isolated mitochondria, increases in vivo mitochondria localization of a BAX-GFP fusion protein, and increases pro-apoptotic activity of the protein (8, 9, 11–13). It is therefore considered that its native structure is a negative regulator of the addressing of BAX to mitochondria and is named the apoptotic regulation of targeting (ART) sequence (8).

At the other end of the protein, the role of the hydrophobic helix α9 as the membrane anchor has been considered obvious for a long time. The homologous sequences in anti-apoptotic BCL-2 and BCL-xL are clearly membrane anchors; their deletion impairs both mitochondria localization and anti-apoptotic activities of the proteins (14, 15), and there were no objective reasons to think that this could be different for BAX. The BAX α9-helix was shown to be able to drive the membrane insertion of a reporter protein (16), but this observation is not absolute evidence because most hydrophobic α-helices are able to do so provided they are long enough. A confirmation of the role of α9 as the primary membrane anchor of BAX was apparently gained when it was shown that its deletion prevented mitochondria localization of a GFP-BAX fusion protein (17). In contrast to these observations, it should be noted that numer-

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* The abbreviations used are: GFP, green fluorescent protein; ART, apoptotic regulation of targeting.
uous reports (18, 19) described the mitochondrial binding and cytochrome c release activity of recombinant BAX (produced in bacteria), which, for technical reasons, was a protein deprived of o9. Also, one report (20) described that overexpression of a C-terminally truncated BAX was able to induce apoptosis.

BAX expression in yeast was first used as a simple functional test for assaying the interactions between BCL-2 family members (two-hybrid system). Most surprisingly, it was found that the LexA-BAX fusion protein was able to kill yeast and that this death was prevented by the co-expression of BCL-2 or BCL-xL-based fusion proteins (21). It was further shown that a C-terminal c-Myc-tagged human BAX not only killed yeast (22, 23) but also induced cytochrome c release, before killing the cells (24). Furthermore, an N-terminal hemagglutinin-tagged mouse BAX was shown to induce other apoptotic hallmarks in yeast (25). Because of the relative easiness of yeast manipulation and the fact that BAX could be expressed independently from the apoptotic network, yeast was used as a tool to test the molecular hypotheses underlying the mechanism of BAX translocation to mitochondria (for review see Ref. 26). Particularly, yeast has proven to be useful to question the actual role of different mitochondrial proteins in their interaction with BAX (27–30).

Concerning the conformational changes underlying the interaction of BAX with mitochondria, the role of the N-terminal end as an ART sequence was confirmed by studies in yeast; like in mammalian cells, the deletion of the first 19 residues of BAX led to a protein having a strong ability to bind to mitochondria and to release cytochrome c (13, 31).

There were discrepancies raised when the role of the o9 was challenged. Expression of an o9-truncated variant in yeast had strictly the same effect as the parental protein BAX-c-Myc (32). Because this could have been a side effect of the c-Myc tag, untagged proteins were assayed. Most surprisingly, although the full-length untagged protein had a poor effect in yeast, the o9-truncated variant remained very efficient in binding to mitochondria, releasing cytochrome c and inducing cell death (31). Two conclusions could be drawn: 1) addition of a (hydrophilic) c-Myc tag increased the efficiency of the protein in yeast; 2) deletion of the o9-helix also increased the efficiency of the protein in yeast. This observation is not linked to the heterologous expression in yeast; deletion of the o9-helix was shown to increase mitochondrial binding and pro-apoptotic activity in gloma cells almost deprived of endogenous BAX (12).

This work investigates further the molecular mechanisms involved in BAX/mitochondria interactions by expressing variants of BAX carrying residue substitutions in the ART sequence or in the vicinity of the o9-helix in yeast. The crucial role of a conformational change of the ART sequence was confirmed, and the data strongly support the hypothesis that o9 is not required for membrane insertion but, on the opposite, is a second ART sequence. In addition, it is shown that helix o9 is required for the interaction of BAX with anti-apoptotic BCL-xL.

### EXPERIMENTAL PROCEDURES

**BAX Variants and BCL-xL**—The mutations in BAX variants and the corresponding mutagenic oligonucleotides are listed in Table I. The BAX gene is a chemically synthesized gene, corresponding to the cDNA of human BAX, but carrying substitutions at the third position of several codons to improve expression in yeast (22). The mutations were introduced by PCR as described previously (31). All genes were entirely sequenced on each strand to verify that additional mutations had not been introduced by the PCR amplification. The genes were introduced in the pYES3 (TRP1 marker) vector (Invitrogen), allowing the regulated expression of the proteins under the control of the galactose-inducible promoter GAL110. The cDNA of human BCL-xL was introduced in the pDP35A (URA3 marker) under the control of the same GAL110 promoter (32). The construction of BAX variants was carried out in bacteria, corresponding to deletions of the 19 N-terminal residues or 20 C-terminal residues, respectively, as described previously (31).

**Yeast Strain and Cultures**—The wild-type haploid strain W303-1B (MATa, ade2, his3, leu2, trpl, ura3) was used in this study. This strain has the advantage of supporting a vigorous oxidative metabolism, thus providing well differentiated mitochondria. Cells were grown aerobically in synthetic minimal medium (0.1% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.1% potassium phosphate, 0.2% Drop-Mix, 0.02% azotrophic requirements) supplemented with 2% D-lactate as a strictly nonfermentable carbon source. Cultures were grown at 28 °C until they reached an absorbance of 0.5 at 550 nm (early exponential growth phase). Then 2% galactose was added to trigger the expression of BAX, and cultures were grown for an additional 14 h. The different variants of BAX were all expressed at high levels showing that the mutations did not strongly decrease BAX synthesis and stability (Fig. 1).

**Mitochondria Preparation**—Cells grown as above were harvested and converted to spheroplasts by a zymolase treatment (ICN). Spheroplasts were disrupted by an osmotic shock and a hand-potter homogenization to preserve the outer mitochondrial membrane, and the mitochondrial fraction was recovered after a series of differential centrifugation (33). Where indicated, mitochondria were incubated (1 mg/ml−1) in the presence of 0.1 m Na2CO3 (pH 11.0) or 0.1% Triton X-100 for 15 min at 4 °C and centrifuged for 15 min at 105,000 × g, and the pellet was treated by SDS-PAGE and Western blot analysis.

**Western Blot Analysis**—50 μg of mitochondrial proteins (treated or not with Na2CO3 or Triton X-100) were solubilized in 0.1 m Tris/HCl (pH 8.8), 2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.004% bromphenol blue, heated for 15 min at 65 °C, and loaded on a SDS-PAGE (12.5% acrylamide/bisacrylamide 30:0.8). After blotting on polyvinylidene difluoride membranes (Problott, Applied Biosystems), they were incubated with primary antibodies (anti-Portp, anti-Bax, anti-C21, anti-BAX antibody BAXC21 (Santa Cruz Biotechnology), 1:500 dilution; directed against the C-terminally truncated mouse BAX, but which also recognizes human BAX) was used to detect the BAX isoform, in comparison to the BAXisoform. Secondary anti-rabbit IgG antibodies coupled to peroxidase (The Jackson Laboratories) were used at a 1:5000 dilution. An anti-yeast porin monoclonal antibody (Molecular Probes; 1:10,000) was used as a loading control. An anti-BCL-x antibody (Calbiochem) was used at a 1:500 dilution to verify the

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**TABLE I**

| Substitution | 5′–3′ sequence of mutagenic oligonucleotides |
|--------------|---------------------------------------------|
| G10V/G11W    | AAC CAA GAG TTG GGG TCC CAA CTT CTT CTT    |
| G12V         | AAG GAG GTG GGG TGG GAA CTT CTT CTT CTT    |
| P9/G13G      | AAG GAG GTG GGG TGG GAA CTT CTT CTT CTT    |
| K189M/K190M  | TGG ACC ATC TGG AAC GAT GGG TGG CTT CTT    |
| K189E/K190E  | TGG ACC ATC TGG AAC GAT GGG TGG CTT CTT    |
| P174D        | GAG TGG GGA GGA GGA GGA CTT CTT CTT CTT    |
| P168A        | GAG CTT GGG GGA GGA GGA GGA CTT CTT CTT    |

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**FIG. 1. Expression of different BAX mutants.** Cells were grown aerobically in lactate-supplemented medium up to 0.5 A550 units. 2% galactose was added to induce the expression of BAX variants during 14 h. 107 cells were washed twice and suspended in 0.5 ml of water. 50 μl of a mixture of 3.5% β-mercaptoethanol in 2 m NaOH was added, and cells were incubated on ice for 30 min. Proteins were precipitated by adding 50 μl of 3 M trichloroacetic acid, and the pellet was resuspended and treated for SDS-PAGE and Western blot analysis with antibodies directed against mitochondrial porin (Portp) and BAX.
expression of this protein after transformation with the plasmids and galactose-driven induction. Peroxidase activity was revealed with ECL/H11001 (Amersham Biosciences), and films at different time exposures were scanned (HP Scanjet 3500c) and quantified with ImageJ software.

**Cytochrome Content**—Mitochondria suspension (5 mg of protein/ml) was divided into the two cuvettes of a dual-beam spectrophotometer (Aminco DW2000). The reference cuvette was oxidized with potassium ferricyanide, and the sample cuvette was reduced with sodium dithionite. Spectra were acquired between 500 and 650 nm allowing us to measure all three mitochondrial cytochromes c, b, and aa₃ (see Fig. 4A for typical recordings) The concentration of cytochromes was calculated from the spectra at 550–540 nm (ε = 18,000 M⁻¹·cm⁻¹) for cytochrome c, 561–575 nm (ε = 18,000 M⁻¹·cm⁻¹) for cytochrome b, and 603–630 nm (ε = 24,000 M⁻¹·cm⁻¹) for cytochrome aa₃, respectively. The advantage of this method is that cytochrome b, which is a membrane protein, is not released nor degraded (34) and serves as an internal control to the experiment.

**RESULTS**

**Mutations in the N-terminal ART Sequence**—The deletion of the 19 N-terminal residues of BAX (containing the ART sequence) results in an increased binding of BAX to mammalian mitochondria in vitro (12). Accordingly, a BAX-GFP fusion protein deprived from the first 17 residues of BAX exhibits a stronger mitochondrial localization in vivo than the full-length BAX-GFP fusion (8). Moreover, high level expression of an N-terminally truncated isoform of BAX (termed BAXᵦ) results in a higher apoptotic efficiency than the expression of the full-length protein in glioma cells (12, 13).

The ART sequence contains a remarkable motif PRGGGP between positions 13 and 18. The RGGG residues are predicted to rotate freely in every direction between the two rigid angles formed by the proline residues. Data obtained by Suzuki et al. (10) showed the very high mobility of ART. A putative movement of ART between two relatively stable positions may be the first event leading to the membrane insertion of BAX, and the

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**Fig. 2. BAX mutants are addressed to mitochondria.** A, typical Western blots obtained on mitochondria isolated from strains expressing different mutants of BAX having different abilities to bind to mitochondria. Western blots against mitochondrial porin (Por1p), an outer membrane protein, serve as a loading control. B, non-saturated Western blots were scanned and quantified with ImageJ software. BAX spots densities (relative to the corresponding porin spots) were normalized to values measured for BAX₦. Experiments are average of 5–10 independent experiments (±S.D.).

**Fig. 3. BAX mutants are inserted in mitochondrial membrane.** Mitochondria isolated from cells expressing different BAX mutants, alone or in combination with BCL-xL, were treated with Na₂CO₃ or Triton X-100 to remove loosely bound or inserted proteins, respectively. All the mutants are insensitive to Na₂CO₃ and sensitive to Triton X-100, showing that they are actually inserted in the membrane.
PRGGGP sequence is a good candidate to be mainly responsible for such a movement. To test this hypothesis, a double substitution P8G/P13G was created, which was expected to increase the mobility of this zone. The gene encoding this BAX variant was introduced in yeast, under the control of a GAL1/10 promoter. Cells were grown aerobically on a nonfermentable carbon source (lactate) until early exponential growth phase ($A_{550} = 0.5$). These conditions ensured an optimal differentiation state of mitochondria. 2% galactose was added to trigger the induction of BAX variant, which was done for 14 h. Mitochondria were isolated from zymolyase-treated cells. The amount of BAX variant addressed to mitochondria was measured by Western blotting, the degree of insertion was measured following treatments of mitochondria with Na$_2$CO$_3$ or Triton X-100, and the extent of cytochrome $c$ release was measured by redox differential spectrometry. Full-length BAX (isoform BAX$^{/H9251}$) and the protein having a deletion of the first 19 residues containing the ART sequence (corresponding to the isoform BAX$^{/H9023}$) were used as negative and positive controls, respectively. An additional negative control was provided by a triple substitution of the glycine residues 10–12 by bigger and more hydrophobic residues (G10V/G11W/G12V).

The proline-to-glycine substitutions at positions 8 and 13 generate a protein that exhibits a high binding capacity to mitochondria (Fig. 2). The protein was not released by Na$_2$CO$_3$ treatment but was solubilized by Triton X-100 (Fig. 3), as expected from a protein inserted in the membrane. This inser-
tion correlates with a high capacity to release cytochrome c (Fig. 4). The properties of the F8G/P13G mutant are more or less the same as those of the BAX isoform, suggesting that the higher mobility imposed to ART has the same stimulating effect as the full deletion of this part of the protein.

Mutations at the C-terminal End—The C-terminal end of BAX is characterized by the presence of two lysine residues at positions 189 and 190. The presence of lysine (or arginine) residues close to the end of the protein is a characteristic of nearly all BCL-2 family members, whether they are pro- or anti-apoptotic, including nonmammalian protein like worm Ced-9 or viral BHRF1 and ORF16 (Fig. 5). Most interestingly, BH3-only members that bear a predicted C-terminal hydrophobic a-helix, like Bim and Bik, do have positive charges at the end of the protein; this is less obvious for BH3-only members that do not have this predicted hydrophobic a-helix, like Bid and Bad, which do have positive charges, but less close to the C terminus.

In order to challenge a role of this conserved characteristics in BAX function, the two positively charged lysine residues were substituted by hydrophobic methionine residues or by negatively charged glutamate residues. Full-length BAXs and the protein deprived from the 20 residues corresponding to the h9-helix and the C-terminal end (BAXΔh9Δ) served as negative and positive controls, respectively.

The K189M/K190M mutant exhibits a marginal binding to mitochondria, close to that of wild-type BAX, and no ability to release cytochrome c (Figs. 2 and 4). On the opposite, the K189E/K190E mutant has a high binding capacity to mitochondria, is inserted into the membrane, and has a high cytochrome c-release activity (Figs. 2–4), showing that the replacement of the two positive charges by negative charges strongly amplifies both the binding capacity and the activity of BAX. The properties of mutant K189E/K190E are not significantly different from that of the mutant deprived of helix h9, showing that the replacement of the two positive charges by negative charges has the same effect on BAX/mitochondria interaction as the complete suppression of the last 20 residues.
exhibited a strong binding to mitochondria (Fig. 2), is inserted (Fig. 3), and has a high capacity to release cytochrome c (Fig. 4).

Mutation of the Proline Residue between α8- and α9-Helices—This zone of the protein contains a proline residue at position 168, which might play a role in the conformational change responsible for the movement of α9 associated to the addressing/insertion process. A recent paper (36) reported that a mutation P168A prevented the translocation of BAX to mitochondria; however, it seemed that, under the conditions of the study (strong overexpression in HeLa cells), BAX-P168A was trapped in the cytosol. It has been observed that, under more moderate conditions of expression in BAX-deficient cells, a BAX-P168V variant exhibited a high capacity of insertion in the outer mitochondrial membrane as well as a strong pro-apoptotic activity.2

The P168A mutant was expressed in yeast. Opposite to the study in HeLa cells, and in accordance with observations in BAX-deficient glioma cells, this mutant was strongly addressed to mitochondria (Fig. 2). It is inserted in the membrane (Fig. 3) and has a strong capacity to release cytochrome c (Fig. 4).

Effects of BAX Mutations for Protection by BCL-xL—Anti-apoptotic proteins of the BCL-2 family, like BCL-xL, are able to physically interact with pro-apoptotic proteins, like BAX. This interaction is thought to involve the BH3 domains of each protein because the ΔGDE mutation in this domain prevents the interaction (21).

Like in mammalian cells, the expression of BCL-xL in yeast is able to prevent the effects of the expression of an active BAX variant (C-terminally c-Myc-tagged BAX); BCL-xL prevents BAX-c-Myc-induced release of cytochrome c and cell death. Similar observations were done for BCL-2. As expected, a C-terminally truncated variant of BCL-xL is much less active, in accordance with the hypothesis that the C-terminal membrane anchor of BCL-xL is required for its anti-apoptotic function. Less expected was the observation that the effects of a C-terminally truncated variant of BAX were not prevented by full-length BCL-xL or BCL-2 (32). This observation suggested that the C-terminal α-helix of BAX was somehow involved in the interaction with anti-apoptotic proteins (23, 32).

This was investigated further in the present study by comparing the protecting effect of full-length BCL-xL over the effects of substituted BAX mutants able to induce a strong release of cytochrome c: P8G/P13G for ART, K189E/K190E for the C-terminal end, T174D for helix α9, and P168A for the loop between helices α8 and α9 (Figs. 7 and 8). The results showed that the addressing of mutant P8G/P13G was almost fully prevented by the co-expression with BCL-xL, as shown previously for isoform BAXY (31). On the opposite, the addressing of mutants T174D, K189E, and P168A was only partly inhibited by the co-expression of BCL-xL; the amount of mitochondrial BAX in the presence of BCL-xL represented 14% of the amount in the absence of BCL-xL for mutant P8G/P13G, whereas it represented 60–70% for the three other mutants (this difference is statistically significant with a p < 0.05).

These mutants thus behave like the mutant deprived from helix α9, for which such a result has been observed previously (32). It should be noted that the remaining mitochondrial BAX mutants in the presence of BCL-xL were still inserted in the membrane, because they were not removed by Na2CO3 and were only solubilized by Triton X-100 (Fig. 3). In accordance with this observation, BCL-xL did not decrease the release induced by the three mutants in the C-terminal part, as this could be expected from the poor effect on their insertion. More surprisingly, the release of cytochrome c induced by the P8G/P13G mutant was not significantly decreased by BCL-xL, despite the dramatic decrease in insertion, suggesting that the low remaining amount of protein in the membrane is still strongly active.

These data confirm that the N-terminal end of BAX is not involved in the interaction with BCL-xL; the complete absence of ART or the substitution of the two proline residues in ART creates active variants that remain sensitive to the inhibition of binding by BCL-xL. On the other hand, the mutations in the C-terminal end, like the complete absence of α9, create active BAX variants that are less sensitive to the effect of BCL-xL.

DISCUSSION

The N-terminal ART Sequence—Data reported in the literature strongly support the hypothesis that the N-terminal end of BAX is a negative regulator of its translocation to mitochondria. Deletion of this sequence increases both BAX physical interaction with mitochondria and pro-apoptotic activity of the protein in all the models and tools studied until now: recombinant protein on isolated mitochondria, expression of a GFP-BAX fusion in mammalian cells, overexpression of BAX in mammalian cells, and heterologous expression in yeast. NMR studies of soluble BAX (10) showed that this zone was poorly defined, because of a high mobility between several positions. The PRGGGP motif localized between positions 8 and 13 is of strong interest; it correspond to a very mobile structure (RGGG) lying between two more rigid links formed by the two proline residues. It was therefore tempting to hypothesize that movements between different conformations were the basis for the conformational change of ART accompanying mitochondria translocation of BAX. Data obtained here gave additional support to this hypothesis: the replacement of the two proline residues by glycine residues, which is predicted to increase the rate of transitions from one conformation to another, strongly increased both the physical interaction of BAX with mitochondria and the activity of the protein. A similar effect of this variant had been observed when it was overexpressed in BAX-deficient mammalian cells (12). The present work shows that conformational changes of ART are able to regulate the addressing process of BAX, independently from a mammalian context, supporting the view that this is an intrinsic property of the protein.

The C-terminal α8-Helix—The actual role of the hydrophobic C-terminal α-helix of BAX as a membrane-targeting signal and a membrane anchor is rarely investigated in the literature, because it is often considered as obvious. The homologous α-helices of anti-apoptotic BCL-2 and BCL-xL, were shown to be membrane anchors because their deletion impairs both mitochondrial localization and anti-apoptotic activity (14, 15). Concerning BAX, the α9-helix is able to target reporter proteins to membranes (16). In addition, deletion of α9 impaired the mitochondrial localization of a GFP-BAX fusion. However, the

FIG. 8. Effect of BCL-xL on the release of cytochrome c induced by BAX mutants. BAX mutants were co-expressed with BCL-xL, and cytochrome c/cytochrome b ratios were measured as in Fig. 4. Average of 4–5 experiments (± S.D.).

2 P. F. Cartron, H. Arokium, L. Oliver, K. Meflah, S. Manon, and F. M. Vallette, submitted for publication.
fact that recombinant BAXΔha9 produced in bacteria was shown by many groups to insert into both mitochondria and liposomes did not raise many doubts about the role of α9 as a membrane anchor.

When expressed in yeast, BAXΔha9 is located in mitochondria and has a potent cytochrome c release activity (31). The same mutant expressed in mammalian cells also has a strong ability to localize to mitochondria and to induce apoptosis (12, 20). Less dramatic alterations were assayed in yeast (31); the replacement of α9 by an unstructured hydrophilic sequence resulted in a very active protein. On the other hand, its replacement by a true membrane anchor, that of BCL-xL, led to a still active protein. On the other hand, its replacement by a true membrane anchor, that of BCL-xL, led to a still active protein.

These data led us to reconsider the actual behavior of α9 during BAX/mitochondria interaction. The T174D-substituted mutant exhibited a strong binding, insertion, and cytochrome c release activity. According to the NMR-based structure of soluble BAX, this residue is located in very close proximity (less than 3.5 Å) to the Glu-69 of the IGDE sequence in the BH3 domain (helix α2) (Fig. 9A). It is thus expected that the introduction of Asp-174 induces a repulsion between the two negative charges with two consequences as follows: 1) the movement of α9 out from the core of the protein, and 2) possibly the exposure of the BH3 domain. Both effects might contribute to the high mitochondria binding and cytochrome c release activity of this mutant.

The crucial role of a movement of α9 is further confirmed by the high binding and activity of the K189E/K190E substituted mutant. The two lysine residues are located in the neighborhood of the Arg-94 residue in the α4 helix. The introduction of a negative charge, namely at position 190, is expected to induce a further proximity of Glu-190 and Arg-94 (Fig. 9B) which, by a counter-movement of the other end of α9, might lead to a structure similar to that expected in the T174D mutant. A recent paper (37) reports that the Akt-dependent phosphorylation of Ser-184 might contribute to stabilize BAX under an inactive form. This observation can be interpreted in different ways, but the location of Ser-184 at less than 5 Å from the Arg-94 and Asp-98 in α4 helix is in accordance with a model where a redistribution of the electric charges caused by the phosphorylation/dephosphorylation of Ser-184 participates in the movement of α9.

Additional recent data suggest a crucial role of the conformation given by the Pro-168 located in the loop just before α9 (36).2 Shinzel et al. (36) observed that mutation of this proline residue impaired the addressing of BAX to mitochondria and suggested that the conformation conferred by this proline is required for an efficient addressing of BAX. The results were interpreted as a requirement for a spatial conformation allowing the insertion of α9; however, they could as well be interpreted as a requirement for a conformation of α9 being able to move correctly or, alternatively, able to confer the adequate exposure to α5 and α6, which were recently demonstrated to be actually responsible for BAX insertion (38). In support to the latter hypothesis, a P168A or a P168V substitution induced a strong activation of BAX both in yeast and in BAX-deficient glioma cells,2 suggesting that, in the absence of endogenous BAX, the conformational change induced by the change of the angle formed by Pro-168 allows the protein to reach a structure close to that able to be inserted into the membrane and to induce the release of cytochrome c. This work and the data from previous reports (20, 31, 32, 35) support the hypothesis that not only is helix α9 not an addressing/insertion sequence but that its movement is also required to allow BAX to be addressed and inserted in the outer mitochondrial membrane, in a manner similar to the N-terminal ART sequence.

The NMR structures of soluble BAX reported by Suzuki et al. (10) allow us to visualize the possible changes concerning Pro-13 and Pro-168; the two structures presented in Fig. 9C are 2 conformations, among 20, determined by NMR. The zones containing Pro-13 and Pro-168 are evidence and show the movements supported by these two residues. In Fig. 9C, structure a, where the N-terminal ART is extended out from the core of the protein, Pro-13 is under a conformation close to that of a β-sheet (ϕ = −62.6°; Ψ = +174.5°). In Fig. 9C, structure b, the conformation of Pro-13 is close to that of an α-helix (ϕ = −68.4°; Ψ = −46.0°). The same changes can be visualized for Pro-168, which is close to an α-helix in Fig. 9C, structure a (ϕ = −62.6°; Ψ = −67.7°) and close to a β-sheet in structure b (ϕ = −65.8°; Ψ = +175.5°); this is accompanied by a change in the structure of the loop between α8 and α9 helices that forms a right angle in structure a and is more flat in structure b of Fig. 9C. Given that substitutions of one of these two proline residues is able to strongly activate BAX/mitochondria interaction, it is likely that conformational changes of these residues are involved in this process, leading to a movement of both the N-terminal ART and the helix α9.

The Interaction of BAX with BCL-xL—In addition to the investigations about the effects of BAX alone on mitochondria, we studied the interactions between BAX and the anti-apoptotic protein BCL-xL. It was reported previously that the absence of BAX-α9 partly prevented the inhibitory effect of BCL-xL on BAX insertion in yeast mitochondria and BAX-induced cell death (23). It was then hypothesized that BAX-α9 played some function in the interaction with BCL-xL. It should be noted that, as expected, the absence of BCL-xL-α8 fully prevented the inhibitory effects of this protein (23, 31) in accordance with the fact that this truncated protein, like BCL-2, is unable to reach a mitochondrial localization (14, 15). Consistent with the hypothesis that BAX-α9 plays some function in the interaction with BCL-xL, the substitutions in the C-terminal part, which affect the conformation of the helix α9, led to
proteins that are less sensitive to the presence of BCL-xL. This is not a characteristic of any mutant able to interact with yeast mitochondria because the substitution in ART (P8G/P13G) led to a protein of which the insertion remained sensitive to the presence of BCL-xL, as it has also been observed for a c-Myc tagged BAX used previously (31). As noted before, from the proposed structure of soluble BAX (10), a part of α9 is localized in close proximity to the BH3 domain, which is widely considered as the domain responsible for the dimerization of BCL-2 family members. It is noteworthy that, from a study in yeast (39), BCL-xL may have at least two inhibitory effects in the process of BAX activation: it would prevent the addressing/insertion step, and also prevent the acquisition of the final conformation able to drive the release of cytochrome c. From experiments realized with a hemagglutinin-tagged BAX in yeast, Policic and Forte (39) suggested that BCL-xL was acting both on the “insertion” step and on the “activation” step. Similarly, the cytochrome c release activity of a c-Myc-tagged BAX is prevented by BCL-xL (31). From the experiments reported here, BCL-xL is able to inhibit the insertion step of a mutant in the N-terminal ART but is not able to inhibit the activation step in this mutant. The inhibitory effect of BCL-xL over BAX insertion probably happens in the cytosol via an interaction between the soluble forms of the proteins, whereas the inhibitory effect over BAX activation probably happens in the membrane phase via an interaction between the integrated form of the proteins; it is thus likely that these distinct inhibitory effects do not involve the same domains of the two proteins, in accordance to the observations that the different active variants of BAX are not inhibited in the same manner by BCL-xL.

It is also interesting to note that a recent work (40) demonstrated that the C-terminal α-helix of BCL-xL is involved in the homodimerization of the protein and the heterodimerization with BAX; this strongly suggests the following: (i) the function of hydrophobic α-helices of BCL-2 family members is not limited to the anchoring function, and (ii) the BH3 domain is not the only domain responsible for oligomerization. This supports the view that the current models based only on the analysis of primary structures do not fully account for the complexity of the functions of the different domains of BCL-2 family members and that complete structural studies will be required to fully understand the regulatory aspects of their functions.

Conclusion—The data reported in this paper, added to previous data obtained both in yeast and mammalian cells, 1) further confirmed the inhibitory function of the N-terminal end of BAX over its interaction with mitochondria, and 2) provided additional evidence against a crucial transmembrane anchor function of α9 which, on the opposite, appears to behave as a second inhibitory sequence over BAX/mitochondria interaction. A recent in vitro study demonstrated the role of α5- and α6-helices as the transmembrane domains of BAX and, in parallel, supported the view that α9 is not required for the membrane insertion of BAX (38). The present study not only is additional evidence against the role of α9 as the “targeting” sequence but further strongly supports the view that it is a second ART sequence involved in the cytosolic retention of BAX in healthy cells, an hypothesis that is supported by the effects of the phosphorylation of Ser-184 (37) and the crucial role of Pro-168 conformation2 in mammalian cells.
Studies of the Interaction of Substituted Mutants of BAX with Yeast Mitochondria Reveal That the C-terminal Hydrophobic α-Helix Is a Second ART Sequence and Plays a Role in the Interaction with Anti-apoptotic BCL-xL

Hubert Arokium, Nadine Camougrand, François M. Vallette and Stéphen Manon

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