The N-terminal End of Bax Contains a Mitochondrial-targeting Signal*

The translocation of Bax, a pro-apoptotic member of the BCL-2 family from the cytosol to mitochondria, is a central event of the apoptotic program. We report here that the N-terminal (NT) end of Bax, which contains its first α helix (H1α1), is a functional mitochondrial-addressing signal both in mammals and in yeast. Similar results were obtained with a newly described variant of Bax called Baxψ, which lacks the first 20 amino acids of Bax and is constitutively associated with mitochondria. Deletion of H1α1 impairs the binding of Baxψ to mitochondria, whereas a fusion of the N terminus of Baxα, which contains H1α1 with a cytosolic protein, results in the binding of the chimeric proteins to mitochondria both in a cell-free assay and in vitro. More importantly, the mitochondria-bound chimeric proteins inhibit the interaction of Baxψ with mitochondria as well as Bax-apoptogenic properties. The mutations of the H1α1, which inhibit Baxα and Baxψ translocation to mitochondria, also block the subsequent activation of the execution phase of apoptosis. Conversely, a deletion of the C terminus does not appear to influence Baxα and Baxψ mitochondrial addressing. Taken together, our results suggest that Bax is targeted to mitochondria by its NT and thus through a pathway that is unique for a member of the BCL-2 family.

Apoptosis is finely regulated by intracellular events, which at its onset, appear to be under the control of members of the BCL-2 family (1). The main site of action of these proteins appears to be the mitochondrion, particularly through the control of the release of apoptogenic factors from this organelle (2, 3). Members of the BCL-2 family can be anti-apoptotic or pro-apoptotic and totally or partially mitochondrial-bound or cytosolic (4). In most cells, one of the crucial and most regulated steps in the implementation of apoptosis is the control of the translocation of Baxα from the cytosol to the mitochondria (4). Anti-apoptotic proteins such as Bcl-2 or Bcl-XL act as inhibitors of Bax function, whereas proapoptotic BH3 only members can either hinder this inhibition (e. g. Bad) or directly activate Baxα (e. g. Bid), which in both cases would promote the association of Baxα with the mitochondrial outer membrane (MOM) (5, 6).

Bcl-2 is anchored into MOM bilayer by a short hydrophobic domain close to the C terminus (CT) with its N terminus (NT) exposed toward the cytosol (7–9). Bcl-2 belongs to a class of membrane proteins called tail-anchored (TA) proteins, which are associated with different intracellular membranes, usually MOM and the endoplasmic reticulum (10). Based on the homology with Bcl-2, it has been proposed that Bax became inserted to mitochondria by its hydrophobic α9 helix located at its C-terminal end (for example, see Ref. 11). Several studies have shown that the Baxα CT is not required for its interaction with mitochondria, whereas others have found it mandatory for Baxα function (12–16). We have observed that a chimeric Bcl-XL construct in which the natural Bcl-XL CT was substituted by that of Baxα was no longer able to associate with mitochondria in the cell-free system, whereas the reverse substitution (i.e. Bcl-XL CT fused to Baxα without CT) led to a massive binding to the organelle (14). Nechustan et al. (11) have shown that unless the serine 184 was properly mutated or deleted, the wild type Bax CT was not a membrane-addressing signal. From these experiments, one could conclude that the membrane-targeting information of Bax is not encoded in its wild type CT. In addition, because Baxα is usually located preferentially in the cytosol and/or in the mitochondria of healthy cells, it does not appear to behave like a classical TA protein (10).

Goping et al. (17) have described the presence of a sequence enclosed in the first 20 amino acid residues regulating Baxα insertion into mitochondria called the ART sequence, an acronym for “apoptosis-regulating targeting” sequence. Recently, we have determined by site-directed mutagenesis that a proline (Pro–13) located within the ART sequence was crucial for the maintenance of the cytosolic Baxα conformation and that its substitution for a valine increased its apoptogenicity (16). We have also discovered that human gliomas expressed a natural variant of Baxα named Baxψ, which lacks the ART sequence (18). Baxψ is highly apoptogenic and is a deletion of its CT, quite remarkably enhanced its association with mitochondria (16, 18). These results suggest that the CT plays a role in the control of the cytosolic conformation of Bax and not in its addressing to the mitochondria (16).

Because of the importance of the translocation of Bax to the mitochondria during apoptosis, we have re-addressed the question of the localization of mitochondrial-targeting sequences in...
Bax α and Bax ψ. We report here that such a sequence is located in the NT of Bax and that inactivating mutations within this sequence affect Bax α- and Bax ψ-intracellular localization and/or proapoptotic function.

MATERIALS AND METHODS

Reagents—Unless specified, all of the reagents used in this study were from Sigma. Monoclonal Bax antibody (clone 4F11) was from Immunotech (Marseille, France). The mitochondrion-selective probe MitoTracker Green-FM (Bachem, Voisins, France). and fluorogenic peptide Ac-DEVD-AMC was from Ozyme, St. Quentin Yvelines, France). Transformer Site-directed mutagenesis using the PCR-based Gateway manufacturer—obtained by site-directed mutagenesis using the PCR-based Gateway manufacturer—was obtained from Molecular Probes (Interchim, Marseille), and fluorogenic peptide Ac-DEVD-AMC was from Bachem (Voisins, France).

Plasmids and Cell Transformations—NT and CT mutants of Bax were obtained by site-directed mutagenesis using the PCR-based Gateway method and were subcloned into a pDEST 12.2 plasmid according to the manufacturer’s instructions (Invitrogen). Red fluorescent proteins (RFP) constructions were subcloned into pDsRed-1N1 and pDsRed-C1 plasmids according to the manufacturer’s instructions (Clontech, Ozyme, St. Quentin Yvelines, France). Transformer Site-directed mutagenesis kit from Clontech was used for internal mutations. Primers used for mutagenesis are listed in Table I. To obtain in vitro translated proteins, RFP constructs were subcloned into pGEM T-easy vector (Promega, Charbonnieres, France) after digestion by SacI/VelI for pDsRed-1N1 and by ApaI/NetI for pDsRed-C1. A Bax-deficient cell line derived from a human Glioblastoma Multiforme (BgDBM) was transfected with the empty vectors or Bax mutants cloned. Plasmid DNA (5 μg) were introduced into 106 BgDBM cells by electroporation (Gene Pulser, Bio-Rad) using 200 V/cm and 250 microfarads. The transfected cells were selected in a medium containing neomycin (250 μg/ml) for 15 min at −20 °C. After saturation with 3% bovine serum albumin in phosphate-buffered saline, the cells were incubated with anti-Bax (1 μg) for 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 a 63 NA Fluotar objective (Leica, Rueil, France). Quantification of the overlay of labeling was done using MetaMorph 4.6 programs (Universal Imaging Corp.). The correlation factor (CF) is a measure of the strength of the relationship between two variables, x (labeling specific for the mitochondria) and y (labeling of constructs of interest), and was calculated as instructed by the manufacturer (www.universal-imaging.com). The RFP was used as a standard for a cytosolic localization. In average, two calculations were performed after analysis of 50 different cells in three independent experiments.

Cell Fractionation—Transfected BgDBM (2 × 106) 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 and then resuspended in CEB (v/v) (250 mM sucrose, 50 mM HEPES, pH 7.4, 50 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 10 μM cytochalasin B, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) as described previously (19). 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 were centrifuged for an additional 15 min at 100,000 × g for 4 °C to obtain the mitochondrial pellets (P13). These supernatants were then centrifuged at 100,000 × g for 30 min in an Airfuge system (Beckman), and the resulting cytosolic fraction was further referred to as the S100. The presence of Bax constructs was detected in mitochondrial pellets and S100 using standard immunoblot methods.

Yeast Experiments—Human Bax variants were cloned at the pme1 site of the pYES3/CT plasmid (Invitrogen) under the control of the GAL1 promoter. Cells were grown aerobically in a synthetic medium supplemented with lactate as a carbon source (0.17% yeast nitrogen base, 0.1% potassium phosphate, 0.5% ammonium sulfate, 0.2% DropMix, and 2% lactate, pH 5.2). The induction of Bax variants was achieved by adding 0.5% galactose, and cells were harvested after different times (see “Results”). Mitochondria were isolated as described previously (20). Cytochrome c release was measured by spectrophotometry of mitochondrial and cytosolic fractions (21). Bax variants and RFP construct association with mitochondrial membrane was determined by alkaline extraction as follows. Mitochondria (1 mg/ml) were suspended quantified with ILab gel program (Signal Analytics, Vienna, VA).

Quantification of Apoptosis—Transfected BgDBM cells (2 × 106) were cultured in the presence or in the absence of doxorubicin (doxo, 20 ng/ml) for 24 h before quantification of apoptosis by measuring the LDH and DEVDase activities (16, 19). The LDH activity was measured using a Cytotox96 assay from Promega as instructed by the manufacturer. After the induction of apoptosis, the cells were washed several times with phosphate-buffered saline 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.

Confocal Analysis—The cells were incubated for 30 min with 5 μg/ml MitoTracker Green-FM at 37 °C and then fixed with 1:1 methanol/acetone for 15 min at −20 °C. After saturation with 3% bovine serum albumin in phosphate-buffered saline, the cells were incubated with anti-Bax (1 μg) for 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 a 63 × 1.3 NA Fluotar objective (Leica, Rueil, France). Quantification of the overlay of labeling was done using MetaMorph 4.6 programs (Universal Imaging Corp.). The correlation factor (CF) is a measure of the strength of the relationship between two variables, x (labeling specific for the mitochondria) and y (labeling of constructs of interest), and was calculated as instructed by the manufacturer (www.universal-imaging.com). The RFP was used as a standard for a cytosolic localization. In average, calculations were performed after analysis of 50 different cells in three independent experiments.

Acellular Assay of Bax Insertion—Mitochondria were prepared from normal rat liver, and cell-free association of Bax with the mitochondria was performed as described previously (16). [35S]Met-Bax associated with isolated mitochondria was analyzed on a SDS-PAGE and scanned with a PhosphorImager (Amerham Biosciences). Insertion of Bcl-2 or of a innermembrane protein was routinely performed in parallel to control the quality of mitochondria. The amount of proteins present in the gel slab was subsequently

Table I

| N- and C-deletions | Bax α S | Bax ψ S | Bax 11–37 S | Bax 1169–192 S |
|-------------------|---------|---------|------------|---------------|
|                   | GGGG ACA AGT TGG TAC AAA AAA GCA GCC TTC ATG GAC GGG GCC GAG | GGGG ACA AGT TGG TAC AAA AAA GCA GCC TTC ATG AAG ACA GGG GCC CTG | GGGG ACA AGT TGG TAC AAA AAA GCA GCC TTC ATG GGG GGG AGG AAC CCC | GGGG ACA CAC TTT GTA CAA GAA AGC TGG GTA TGC CAG GTC GGC GTC CCA |
|                   | 1–19 S  | 20–37 S | 169–192 S  | 169–192 AS    |
|                   | ATGGGAGGGCCGGCACTGG | ATGGGAGGGCCGGCACTGG | ATGGGAGGGCCGGCACTGG | ATGCCCTCGTGAGCCCTGG |
|                   | 1–19 AS  | 20–30 AS | 169–192 AS | 169–192 AS    |
|                   | AGAAGACGCCGGCGGCTG | AGAAGACGCCGGCGGCTG | AGAAGACGCCGGCGGCTG | AGAAGACGCCGGCGGCTG |
|                   | *S184    | A24R    | L26G       | L26G          |
|                   | TCAGCCACTTCCTGACAGTGAGGGGGGCTG | ATGAAGACGCCGGCGGCTG | ATGAAGACGCCGGCGGCTG | ATGAAGACGCCGGCGGCTG |
|                   | Plasmid mutation | pDEST12.2 NheI |
|                   | CGTGCAGGACGTGGGAAAACCTTTCTGTGG |

2 P.-F. Cartron, M. Juin, P. H. Oliver, K. Melfah, and F. M. Vallette, manuscript in preparation.
CEN6FUGATION AT 100,000 g FOR 1 HR ON ICE, MEMBRANES WERE RECOVERED BY A 15-MIN INCUBATION ON ICE, AND IMMUNOBLOTS WERE PERFORMED AS DESCRIBED BY OLIVER ET AL. 

RESULTS

A Sequence Located in the First 39 Amino Acids of Bax Regulates Its Targeting to the Mitochondria—We have previously shown that the deletion of Bax CT does not affect the association of Bax α with mitochondria but enhances that of α variant, which lacks the first 20 amino acids of the Bax α sequence (16). Deletion of the first 20 amino acids of Bax α structurally corresponds to a variant called Bax ψ, which is encoded by a transcript distinct from that of Bax α (18). To further explore the association of Bax ψ with mitochondria, we prepared a series of NT deletion constructs (Fig. 1A) and analyzed in a cell-free system the binding to mitochondria of the corresponding in vitro translated proteins (Fig. 1B). As already shown by several reports (14, 17), the association of Bax α with mitochondria in the cell-free system is extremely low but can be enhanced by the deletion of the first 19 amino acids. Conversely, a construct lacking the first 37 amino acids of Bax α (Bax-(Δ1–37)) does not bind to mitochondria (Fig. 1B).

We next examined the intracellular localization of these constructs after transient transfection in BdGBM.2 As shown in Fig. 2A, a similar amount of Bax constructs was produced by the expression of the various transgenes. We performed cell fractionation to assess the subcellular localization of the constructs analyzed above. As shown in Fig. 2B, the results obtained were consistent with the acellular assay as Bax ψ was found essentially in the mitochondrial pellet (mito) and Bax α-(Δ1–37) in the cytosolic fraction (S100). Bax α and Bax-(Δ169–192) were found in both compartments, however, with a predominant cytosolic localization (Fig. 2B). Confocal microscopy was used as another technique to confirm the mitochondrial localization of these different constructs. As illustrated in Fig. 2C, the product of the Bax α transgene was essentially cytosolic, whereas that of Bax ψ was predominantly mitochondrial-bound as reported previously (18). We quantified the correlation between labeling of mitochondria with that of Bax constructs using a MetaMorph program and compared it with that of the cytosolic protein RFP (see “Materials and Methods”).

As described in the cell-free assay and in the subcellular fractionation, the deletion of the first 18 amino acids of Bax ψ (i.e. Bax α-(Δ1–19)) abrogates the binding of the proapoptotic protein to mitochondria in vitro, which exhibit a correlation factor of a cytosolic protein (i.e. CF = 0.6). On the other hand, Bax ψ was mitochondrial (CF = 1), and Bax α and Bax-(Δ169–192) with a CF of 0.79 and 0.78, respectively, were localized in both compartments (Fig. 2D). Thus, based on the acellular assay, the confocal analysis, and the cell fractionation results, we conclude that the first 18 amino acids of Bax ψ contained a sequence involved in Bax targeting to mitochondria.

As reported previously (16), the mere expression of Bax in the BdGBM cells is not sufficient to trigger cell death (cf cell morphology in Fig. 2C); thus, we quantified the death-inducing capacity of the different constructs after treatment with 20 ng/ml doxo for 24 h. Cell death was assessed using both the release of the cytosolic enzyme lactate dehydrogenase (LDH) into culture and the direct measure of caspase activity in cell extracts (see “Materials and Methods”). As shown in Fig. 3, both LDH release and DEVdase activities were enhanced in cells transfected with Bax α and Bax ψ although with different amplitudes as described previously (18). On the other hand, in contrast with another report (22), little or no induction of cell death was observed with the Bax-(Δ1–37) construct (Fig. 3). It is noteworthy that a deletion of the putative trans-membrane domain of Bax (CT = amino acids 169–193) had no effects on both the subcellular localization and apoptogenic activity Bax α (Figs. 2 and 3).

The α1 Helix of Bax Alone Is Sufficient to Target a Cytosolic Protein to the Mitochondria—According to NMR studies (23), the first 18 amino acids of Bax ψ (i.e. amino acids 20–37 of Bax α) contains an amphipathic α helix (Hα1). Numerous studies have shown that such α helices are present in membrane-targeting sequence (24).

We have generated chimeric proteins by fusion of sequences derived from Bax α NT to the N terminus of the cytosolic RFP (see “Materials and Methods”) and analyzed the binding of these constructs in the cell-free system using in vitro translated chimeric proteins. We also constructed chimeric proteins in which the wild type α9 helix and a mutated form of this sequence (i.e. deletion of the serine 184, Bax-(ΔS184)) were fused to the CT of the RFP. These constructs were similar to those described by Nechustan et al. (11) with the exception that the RFP was used instead of the green fluorescent protein. We
confirmed using the cell-free system their original observation because Bax \( \text{CT} \) did not lead to the association of the RFP with the mitochondria, whereas the Bax-(\( \Delta S184 \)) mutant exhibited higher affinity for mitochondria (Fig. 4A). As also shown in Fig. 4A, the first 37 amino acids of Bax \( \alpha \) triggered the association of the RFP with the mitochondria in contrast to that observed with the RFP alone, which remained cytosolic. On the other hand, H\( \text{9251} \) alone (Bax-(20–37)) and, to a lesser degree, the sequence encompassing the first 19 amino acids of Bax \( \text{H9251} \) were promoting an efficient association of the RFP with mitochondria when compared with the chimera formed from the fusion of Bax \( \text{CT} \) to the CT of RFP (i.e. Bax-(169–192)). However, it should be noted that the Bax-(1–37)-RFP construct exhibited interesting behavior as its association with mitochondria was an intermediate between that of Bax-(20–37)-RFP and that of Bax-(1–19)-RFP (Fig. 4B). These results suggest that the H\( \text{9251} \) (Bax-(20–37)) is more efficient to target Bax to mitochondria than the full-length sequence (Bax-(1–37)) and the ART sequence (Bax-(1–19)), independently of the presence of other Bax domains. Interestingly, none of these constructs were resistant to alkali treatment, which suggests that the NT of Bax is a targeting but not a membrane-anchoring signal. These results were confirmed by cell fractionation (Fig. 4B) and confocal microscopy/MetaMorph analysis (Fig. 4, C and D). The Bax-(1–19)-RFP showed a more cytosolic pattern (CF = 0.82) than the Bax-(20–37)-RFP, which is mainly associated with mitochondria (CF = 1) (Fig. 4, B–D). Quite interestingly, the
Bax-(1–37)-RFP fusion protein exhibited a mixed cytosolic and mitochondrial localization (CF = 0.85) reminiscent of Bax α subcellular localization in these cells (Fig. 4, B–D). The wild type RFP-Bax CT and the Bax-(ΔS184) mutant (see above) behaved as described by Nechustan et al. (11) as the wild type CT construct was mostly cytosolic (CF = 0.7) and the mutated construct was mainly mitochondria-bound (CF = 0.95) (Fig. 4, B–D).

**Competition between Bax ψ and Bax NT-RFP Constructs for Binding to Mitochondria**—We next analyzed, using the cell free assay, the binding of Bax ψ to mitochondria in the presence of increasing concentrations of the different Bax NT-RFP constructs. As shown in Fig. 5A, the addition of Bax-(1–19)-RFP did not interfere with the binding of Bax ψ to mitochondria in the cell-free assay. Conversely, both Bax-(1–37)-RFP and Bax-(20–37)-RFP constructs inhibited this association although with an efficiency that appears to be related with the relative affinity of these constructs for the organelle in the cell-free system (Fig. 5A). The specificity of the interference of the association of Bax with the Bax NT-RFP constructs was assessed by the coincubation of the Bax CT constructs: the wild type RFP-Bax CT and the Bax-(ΔS184) mutant. As illustrated in Fig. 5B, neither construct competed for Bax ψ binding, although RFP-Bax-(ΔS184) association to mitochondria was roughly similar to that observed for Bax-(1–37)-RFP.

Because acellular analysis suggested that the NT-RFP constructs could exhibit a dominant negative function toward Bax, we analyzed stably transfected cells in RFP constructs, the sensitivity to apoptosis upon transient expression of Bax α or Bax ψ. We used cells expressing the same amounts of constructs as quantified by fluorescence of the RFP (Fig. 6A). These cells transfected with either Bax α or Bax ψ were treated with doxo, and cell death was quantified both by assessing the activity of LDH in the culture medium and the cellular DEV-
Dase activity. Compared with control, the coexpression of Bax-(1–37)-RFP and Bax-(20–37)-RFP constructs inhibited Bax α and Bax ψ sensitization to doxo-induced apoptosis, whereas Bax-(1–19)-RFP constructs had only a slight effect (Fig. 6, B and C). It should be noted that the expression of RFP-Bax CT constructs including that of the mitochondria bound RFP-Bax-(ΔS184) did not affect Bax function in these experiments.

Altogether, these results suggest a competition between Bax Ho1-RFP constructs and Bax α or Bax ψ. This competition is likely to occur by the inhibition of the insertion of Bax α or Bax ψ into mitochondria, and this was confirmed by confocal microscopy (data not shown).

**Mutations That Affect Bax ψ Binding to Mitochondria and Its Proapoptotic Activity Also Affect Bax α**—As part of a mutagenesis screening of Bax NT, we substituted Ala-24 for Arg (A24R) and Leu-26 for Gly (L26G, Bax α numbering) in both Bax α and Bax ψ (Fig. 7A). The L26G substitution provoked a break in the Ho1, and the A24R reinforced the basic character of the region surrounding the Ho1, two cardinal points of mitochondrial presequences (24). As shown in Fig. 7B, in both cases, the acellular association of *in vitro* translated Bax α and Bax ψ mutants with mitochondria was significantly decreased, although only slightly for the A24R substitution (Fig. 7B). Interestingly, the effect of the Ho1 disruption (L26G) was more important in Bax ψ than in Bax α, and the additional change in the Ho1 (double substitution L26G/L27V) did not significantly enhance that of the single L26G mutation (Fig. 7B). In both cases, a disruption of the Ho1 had a greater effect on the binding of Bax than a change in the overall positive charge of the molecule. The latter result suggests that the amphipathic nature of the Ho1 is more important for mitochondrial association than its basic character.

We next analyzed by cell fractionation and confocal microscopy the subcellular localization of these constructs in BdGBM cells. The mutations that affected Bax α or Bax ψ binding to mitochondria in the cell-free assay also affected its intracellular localization, and this was particularly striking for Bax ψ in which the L26G substitution almost abolished its constitutive binding to mitochondria (Fig. 7, C–E).

We have analyzed the influence of the expression of these mutants on the apoptotic response induced by doxo (cf “Materials and Methods”). As shown in Fig. 8, the A24R substitution had little or no influence on Bax α or Bax ψ proapoptotic activities. Conversely, in both cases, the L26G mutants nullified the proapoptotic activities of the Bax variants (Fig. 8), a result consistent with the inhibition of its addressing to mitochondria.

**Effect of the Mutations of Bax α NT in Yeast**—It has been reported that Bax-induced growth arrest in yeast was preceded by its binding to mitochondria and, as in mammals, by an increase in the release of cytochrome c into the cytosol (21). We have investigated the effect of a deletion of Bax NT and of mutations in the Ho1 on both *in vivo* binding of Bax to yeast mitochondria and cytochrome c release-promoting activity. The deletion of the first 19 amino acids of Bax α enhanced both the binding of the protein to mitochondria (data not shown) and cytochrome c release (Fig. 9). As observed in human mitochondria, the A24R substitution affected both the binding of Bax-(Δ1–19) to mitochondria (data not shown) and its cytochrome c release activity (Fig. 9). However, contrary to what was observed in human cells, the L26G mutation alone did not affect Bax-(Δ1–19) activity and the additional mutation of the adjacent Leu (L27V) was required to decrease its cytochrome c release activity (Fig. 9). These results suggest that the interaction of the Ho1 with mitochondria is similar but not identical in yeast and in mammalian cells.
released into the culture medium. monitored by measuring the cellular DEVDase activity and the LDH

These cells were stably transfected with the RFP constructs, were 

parental mocked transfected cells is indicated by the 

chosen because they expressed similar amounts of RFP. Staining of the 

sensitization to doxo-induced apoptosis 

Bax (20–37) construct could by acting as a BH3-only protein trigger apoptosis not by itself but through the activation of endogenous Bax, a feature impossible in Bax-deficient cell line. 

The amino acids 20–37 corresponds to the Ha1 of Bax α and Bax ψ (23) and, as such, is a probable candidate for an import signal of Bax α and Bax ψ to mitochondria. To test this hypothesis, we have constructed several chimeric proteins in which different sequences of the NT with or without the Ha1 were fused to the N-terminal of the RFP. These constructs behaved both in a cell-free system and in vitro like the NT truncated Bax constructs described above. The Bax-(1–37)-RFP and the Bax-(1–19)-RFP constructs were partly cytosolic, whereas the Bax-(20–37)-RFP was found mainly to be associated with the mitochondria (Fig. 4). Conversely, the RFP-Bax-(169–192) fusion protein, which contained the entire α9 helix, was unable to address the RFP to mitochondria unless Ser 184 was deleted (Fig. 4). The insertion of the mitochondrial-bound Bax NT-RFP constructs was found to be alkaline-sensitive (Fig. 4), suggesting that this segment was not a transmembrane domain but rather a targeting sequence. This result is consistent with the fact that a trans-membrane domain usually consists of 20–25 non-polar amino acids (24) and thus is significantly longer than Ha1. Because Bax localization was not affected by the deletion of the CT, the membrane-anchoring domains of Bax have to be localized elsewhere. Preliminary results suggest that in Bax ψ, the α5 and α6 helices, which are the putative pore-forming domains of Bax, are involved in this process as already shown by Nouraini et al. (28). Interestingly, several outer membrane proteins such as TOM22 have been shown to possess physically distinct import and membrane anchor sequences that regulate their interaction with mitochondria (29).

The fact that the Bax NT-RFP constructs, which contain the Ha1, can compete for Bax insertion in the cell-free assay (Fig. 5) and act as dominant negative in vitro (Fig. 6), strongly suggesting that this targeting signal involves common mitochondrial or cytosolic receptor(s), remains to be determined. Preliminary results suggest that protease-sensitive component(s) located in the outer membrane are involved in the docking of Bax ψ to mitochondria. Experiments are currently

DISCUSSION

Single membrane-spanning proteins of the MOM can be inserted by their N terminus (e.g., TOM70) or by their C terminus (e.g., Bcl-2) (25). Because of sequence homology, the protein Bax has been thought to belong to the class of TA proteins (1). The two cardinal features of TA proteins are as follows: (i) a distribution between several intracellular compartments such as mitochondria, endoplasmic reticulum, and the Golgi apparatus and (ii) the fact that the targeting information of the protein is encoded in the CT (10). Bax fails to comply with these two points as it is usually predominantly cytosolic and/or mitochondrial in healthy cells and because its CT cannot support the targeting of a reporter protein (this study and 11, 14). Indeed, we have observed that the translocation of Bax upon the induction of apoptosis to mitochondria appears to occur independently of its CT (16, 19). In addition, contrary to what was found for Bcl-2 (7–9), the nature of the sequence located in the Bax CT could influence its proapoptotic activity without affecting its intracellular localization (11, 19). Thus, it has been proposed that the Bax CT could be involved in protein-protein interactions that could regulate its proapoptotic function (26). Goping et al. (17) have shown that the Bax NT contained a sequence involved in the retention of the whole protein in the cytosol. The deletion of this sequence called ART induced a massive translocation of Bax from the cytosol to the mitochondria (16, 17). It has been proposed that the cytosolic conformation of Bax was secured by the interaction between both extremities or by the CT with the BH3 domain and that its translocation to mitochondria involved a conformational change leading to the exposure of the Bax NT (11, 16–17). Interactions among Bax molecules or with other proapoptogenic proteins such as Bid appear to be crucial to induce a change in the conformation of Bax and/or its interaction with mitochondria (5–6). However, these changes seem to be necessary but not sufficient to induce apoptogenic outer membrane permeability (27).

We have recently described a new variant of Bax α, Bax ψ, which is constitutively associated with mitochondria (18). A deletion of the CT of Bax ψ enhanced both its mitochondrial association and its apoptogenic properties, suggesting again that the CT of Bax was mainly involved in the control of a cytosolic conformation (16). Thus, this study investigates the presence of a mitochondrial targeting signal of Bax in the NT. We constructed a series of NT-truncated variant of Bax α and Bax ψ and found that a sequence encompassing amino acids 20–37 (Bax α numbering) was important for the association of Bax with mitochondria both in a cell-free assay and in vitro (Figs. 1 and 2). The deletion of this sequence in Bax α inhibits both its constitutive mitochondrial localization and its proapoptotic activity (Figs. 1 and 2). Our results are in opposition with that of Wood and Newcomb (22), which show that the deletion of this particular sequence enhanced its proapoptotic function. However, in the latter case, the transfection was performed in Bax-expressing cell lines, and thus, it is conceivable that the Bax-(Δ1–37) construct could by acting as a BH3-only protein trigger apoptosis not by itself but through the activation of endogenous Bax, a feature impossible in Bax-deficient cell line.

Fig. 6. Bax-(1–37)-RFP and Bax-(20–37)-RFP inhibit Bax α and Bax ψ sensitization to doxo-induced apoptosis in vitro. A, the expression of the wild type RFP and the Bax-RFP constructs in stably transfected BdGBM was determined by flow cytometry as described under "Materials and Methods." Clones used in the experiments were chosen because they expressed similar amounts of RFP. Staining of the parental mock-transfected cells is indicated by the thin line in A. These cells were stably transfected with the RFP constructs, were transiently transfected by Bax ψ (B) or Bax α (C), and treated with doxo as described under "Materials and Methods." Apoptotic amplitude was monitored by measuring the cellular DEVDase activity and the LDH released into the culture medium.
FIG. 7. Mutations in the Ho1 affects Bax association with mitochondria in a cell-free and in vitro assay. 

A, schematic representation of mutants of the Ho1 present in Bax α and Bax ψ. B, cell-free association of Bax α and Bax ψ and mutants of the Ho1 with purified rat liver mitochondria. The binding of Bax α and mutants was performed as described under “Materials and Methods.” The A24R (Bax α numbering) substitution has a slight but significant influence on Bax α binding to mitochondria. On the other hand, the substitution of L26G with or without the additional substitution of L27V impaired the binding of both Bax α and Bax ψ. Histograms show the amount of protein (fmol) present in the mitochondrial pellet. The data shown and means ± S.D. were obtained from at least four independent experiments. C, subcellular localization of Bax constructs by cell fractionation. BdGBM cells expressing Bax Ho1 mutants were fractionated, and the cytosolic fraction (S100) and the mitochondrial pellet (mito) were analyzed as shown in Fig. 2B. Western blots shown are representative of three different experiments. D, confocal microscopy analysis of Ho1 mutants subcellular localization. The above mutated Bax α or Bax ψ proteins were transiently expressed in BdGBM cells and analyzed as in described under “Materials and Methods.” Magnification = ×60. E, correlation factor between a mitochondrial probe and Bax α or Bax ψ construct localizations. The correlation function was calculated as in Fig. 2D. Means ± S.D. were calculated from the analysis of 50 different cells for each construct.
means apoptogenic properties. Quantification of apoptosis triggered by Bax mutants expression in BdGBM is shown. Cells transfected by the different Bax H1 mutants were treated with doxo for 24 h before measuring the DEVDase activities in the cells and the LDH activity in the culture media ("Materials and Methods"). The data shown and ± S.D. were obtained from at least four independent experiments.

being carried out to quantify and to identify these components. The importance of the NT-H1 in Bax targeting and function was further emphasized by the observation that mutations that affected Bax binding in the cell-free system (Fig. 7) also abolished its subcellular localization and the subsequent induction of the demolition phase of apoptosis (Fig. 8). Similar features were found in Saccharomyces cerevisiae, although additional mutations were required to abolish the addressing property of the H1 (Fig. 9). These results strengthen the idea that the H1 fulfills a basic role in Bax interaction with mitochondria.

Interestingly, the H1 exhibit some structural homologies with the BH4 domain of Bcl-2 and Bcl-XL, although it has been clearly demonstrated that Bax lacks such a "functional BH4-like" domain (30). Indeed, the mutations reported in this work, which affect the binding of Bax to mitochondria (A24R and L27V), were primarily intended to introduce amino acids present in Bcl-2 and Bcl-XL sequences to reinforce the BH4-like aspect of the H1 (30). We show here that these mutations impair Bax proapoptotic function by interfering with its addressing to mitochondria and not by introducing a BH4-like anti-apoptotic function. Nechustan et al. (11) have shown that structural changes in Bax, which accompany the induction of apoptosis, result in the exposition of the H1 tail, and three-dimensional analyses of the detergent-induced changes in the conformation of Bax indicate that the NT (i.e. until the H2) is still flexible and solvent-exposed in Bax oligomers (23). Thus, the H1 is probably still capable of addressing Bax oligomers formed during the onset of apoptosis to mitochondria.

We propose that the apoptotic-induced conformational changes in Bax frees the H1 from its interaction with the rest of the molecule, thus enabling the addressing of Bax to mitochondria through this helix. This association is necessary but not sufficient to trigger mitochondria outer membrane permeability, which can only be achieved through an "apoptogenic" Bax insertion within MOM. The precise mechanism of Bax insertion and especially the role of other proteins of MOM and/or the involvement of other domains of Bax in this insertion remain to be established.

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Fig. 8. The mutants of H1, which affected the binding of Bax in the cell-free system inhibited both Bax α (A) and Bax ϕ (B) apoptotic properties. Quantification of apoptosis triggered by Bax mutants expression in BdGBM is shown. Cells transfected by the different Bax H1 mutants were treated with doxo for 24 h before measuring the DEVDase activities in the cells and the LDH activity in the cell-free system inhibited both Bax binding in the cell-free system (Fig. 7) also abolishing its subcellular localization and the subsequent induction of the demolition phase of apoptosis (Fig. 8). Similar features were found in Saccharomyces cerevisiae, although additional mutations were required to abolish the addressing property of the H1 (Fig. 9). These results strengthen the idea that the H1 fulfills a basic role in Bax interaction with mitochondria.

Fig. 9. Cytochrome c release induced by mutants in the H1 in S. cerevisiae. Mitochondria were isolated from Bax H1 mutant-transformed yeast, and cytochrome c content was measured by spectrophotometry (see "Materials and Methods"). Results are expressed as the mean ± S.D. of at least three independent experiments.

Bax Targeting to Mitochondria