Distinct Domains Control the Addressing and the Insertion of Bax into Mitochondria*§

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The translocation of Bax from the cytosol into the mitochondrial outer membrane is a central event during apoptosis. We report that beyond the addressing step, which involves its first α-helix (hα1), the helices α5 and α6 (hα5α6) are responsible for the insertion of Bax into mitochondrial outer membrane bilayer. The translocation of Bax to mitochondria is associated with specific changes in the conformation of the protein that are under the control of two prolines: Pro-13, which controls the unfolding of hα1, and Pro-168, a proline located immediately before the hydrophobic carboxy-terminal end (i.e. helix α9, hα9), which controls the disclosure of hα5α6. An additional step, the disruption of an electrostatic bond formed between Asp-33 (hα1) and Lys-64 (hB3), allows the mitochondria addressing of Bax. We conclude that, although the intramolecular interactions of hα1 with the hB3 region control the addressing of Bax to mitochondria, the Pro-168 is involved in the control of its membrane insertion through hα5α6.

The BCL-2 family of proteins is a central regulator of apoptosis, because these proteins are the final integrators of most death signals (1). This family is divided into two groups: the first with anti-apoptotic/pro-survival properties such as Bcl-2, Bcl-xL, Mcl-1, and the second with pro-apoptotic/death-promoting properties such as Bax and Bak (1). These two groups of proteins share some degrees of homology, which are restricted to three domains called BH1, BH2, and BH3. Associated with this family are proteins like Bid or Bad, with a homology with Bcl-2 limited to the BH3 region only and hence called BH3-only proteins (BOP) (2, 3). These proteins activate or sensitize to apoptosis by interacting with Bcl-2 family members: the multidomain pro-apoptotic proteins Bax and Bak can be activated directly by BOP-like Bid, whereas another BOP-like Bad activates apoptosis by binding to and thus inactivating anti-apoptotic proteins (2, 3). The main function of Bax and Bak appears to be the induction of permeabilization of the mitochondrial outer membrane (MOM), through a mechanism that remains obscure (4). According to the rheostat model (5), interactions between anti- and pro-apoptotic proteins directly or indirectly regulate apoptosis through the permeabilization of MOM.

Bax is a globular protein, present in the cytosol of resting cells, in an inactive state. However, in response to apoptotic stimuli, Bax undergoes specific conformational changes, which allow its targeting/insertion into MOM (1). Bid induces conformational changes in Bax that cause its dimerization and integration into mitochondrial membranes (6, 7). Structural studies of Bax (8) show that this protein consists of nine helices (α) that are able to form a hydrophobic pocket into which a BH3 peptide from another protein may bind. These studies also showed that the hα5α6 of Bcl-xL, Bcl-2, and Bax are structurally similar to the pore-forming domain of the colicins A/E1 and diphtheria toxin (9). The latter result suggests that both proteins could be able to function as membrane ions channels and as such facilitate MOM permeabilization during apoptosis (9). Consistent with these results, it has been shown that Bax formed ionic channels in artificial membranes and that the electrical activity was indeed associated with the hα5α6 (10, 11).

The lack of mitochondrial integration of Bax in the absence of a death signal is correlated with a repression imposed upon the targeting signal of Bax by the NH2-terminal (NT) domain (i.e. the first 20 amino acids of Bax α) called ART for apoptosis-regulating targeting sequence (12). It has been postulated that both the NT and carboxyl termini (CT) of Bax cooperated to maintain the inactive cytosolic conformation of Bax (12, 13). We have recently reported that the hα1 of Bax, which immediately follows the ART domain, carries a MOM targeting signal, because this sequence causes the addressing to mitochondria of a cytosolic protein and this construct, unlike a similar construct with ho9, inhibits the association of Bax with mitochondria (14). We and others have shown that deletion of the hydrophobic CT of Bax (i.e. ho9) did not modify the intracellular localization in the presence of the ART domain (15–19), whereas a deletion of both sequences enhanced Bax binding to MOM (18, 20). These results suggest that Bax NT and not its CT are involved in Bax translocation to mitochondria. Conversely, Schinzell et al. (21) have recently reported that a Pro located near the CT of Bax (Pro-168) was crucial in directing Bax localization to mitochondria and could allow a ho9-mediated targeting of a reporter cytosolic protein.
Because Bax-induced targeting to, insertion into, and permeabilization of MOM is a crucial step in the implementation of many forms of apoptosis, we have investigated the role of the helices α 1, 9, 5, and 6 in the acquisition of mitochondria-bound Bax conformation.

EXPERIMENTAL PROCEDURES

Materials—Unless specified, all reagents used in this study were from Sigma (St. Louis, MO). The following antibodies were used: monoclonal anti-Bax 2D2 and 6A7 antibodies from R&D Systems (Clontech, Palo Alto) and monoclonal anti-cytochrome c antibodies were obtained from Molecular Probes (Interchim, Montluçon, France). The following antibodies were used: monoclonal Bax antibody (clone 4F11) was from Immunotech (Cergy, France), and Fluorescent Alexa 568TM- and Alexa 468TM-conjugated secondary antibodies were purchased from Life Technologies (Clontech, Palo Alto, CA). The mitochondrial-selective probe Mitotracker GreenTM was from Molecure (Paris, France), and Fluorescent Alexa 568TM- and Alexa 468TM-conjugated secondary antibodies were purchased from Life Technologies (Clontech, Palo Alto, CA). The following antibodies were used: monoclonal Bax antibody (clone 4F11) was from Immunotech (Cergy, France), and Fluorescent Alexa 568TM- and Alexa 468TM-conjugated secondary antibodies were purchased from Life Technologies (Clontech, Palo Alto, CA).

A Bax-deficient cell line derived from a human glioblastoma multiforme (BdGBM) treated with a Bak antisense (14) was transfected with the empty vector (pDEST12.2 or pDEST3.2.) or Bax mutants cloned into this vector. Plasmid DNAs (5 μg) were introduced into 10⁶ BdGBM-Bak (-) cells by electroporation (GenePulsar, Bio-Rad) using 200 V/cm and 250 microfarads, the transfected cells were selected in a medium containing neomycin (250 μg/ml) for 48 h. After selection, the bulk of transfected cells were used in in vitro experiments to avoid clonal bias as described previously (16).

Accleral Assay of Bax Insertion—Mitochondria were prepared from normal rat liver as described previously (24). Of note, the presence of endoplasmic reticulum markers such as GRP 78 was routinely checked by immunoblots prior to any further experiments, and in most cases, no or little contamination by endoplasmic reticulum was found in our preparations. Cell-free association of Bax with the mitochondria was performed as described previously (23): briefly, [35S]Met (Amersham Biosciences)-labeled proteins were synthesized from cDNAs using the TnT-coupled Transcription/Translation system from Promega (Madison, WI) to obtain IVT Bax. The post-translational insertion of IVT proteins into mitochondrial membranes and the subsequent alkaline treatment (0.1 M Na2CO3, pH 11.5) of the mitochondria were performed in a standard import buffer as described previously (23). IVT proteins bound to untreated Bax-deficient mitochondria were recovered after centrifugation of the incubation mixture for 10 min at 4°C and at 8000 g in the pellet. [35S]Met-IVT Bax associated with isolated mitochondria was analyzed in a SDS-PAGE and scanned with a PhosphImager (Molecular Dynamics). The amount of proteins present in the gel was quantified with the IP Lab gel program (Signal Analytics, Vienna, VA) and expressed as a percentage of initial input. To analyze the release of mitochondrial apoptogenic factors from mitochondria, the supernatant derived from the latter accleral assay was incubated...
together with a non-apoptotic cellular extract, and the DEVDase activity was measured in these extracts as described before (23). For the release of cytochrome $c$ (cyt $c$), $20 \mu l$ of supernatant from the acellular assay was analyzed by immunoblotting and quantified with the IP Lab gel program. In all experiments, the aggregation of proteins was controlled by incubating the Bax constructs with non-relevant antibodies in the absence or presence of mitochondria. Of note, we never found a significant amount of proteins aggregating under our conditions (data not shown). Binding of IVT Bax to Bcl-2 or its stimulation by p13-tBid was performed as described previously (14).

Quantification of Apoptosis—BdGBM cells ($2 \times 10^5$ cells), transfected with the different Bax constructs, were cultured in the presence or in the absence of doxorubicin (DOX, $2 \mu g/ml$) for 16 h before quantification of apoptosis by measurement of DEVDase activities. Briefly, the cells were washed several times with phosphate-buffered saline, plated in 96-well plates, and then the caspase-3 fluorogenic substrate (Ac-DEVD-AMC) was added in the presence of 0.01% Triton X-100 and enzymatic activity was quantified in a fluorometer over a period of 1 h ($t_1$), whereas the activity was in a linear range. The activities were calculated from the differences measured between $t_1$ and $t_0$ and thus were not affected by the different backgrounds.

Confocal Analysis—For laser confocal microscopy analysis, the cells were incubated for 30 min with 5 $\mu g/ml$ MitoTracker Green$^{TM}$ at 37 °C then fixed with 1:1 methanol/aceton for 15 min at −20 °C. After saturation with 3% bovine serum albumin in phosphate-buffered saline, the cells were incubated with anti-Bax and anti-cyt $c$ antibodies for 1 h at 37 °C. Cells were extensively washed, and then the conjugated secondary antibodies were added for 1 h at 37 °C. The weak auto fluorescence due to doxorubicin, which was mostly located in the nuclei of the treated cells, did not interfere with our analyses. Images were collected on a Leica TCS NT microscope with a 63 × 1.3 NA Fluotar objective (Leica, France). Quantification of the overlay of labeling was done using a MetaMorph 4.6 program (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 specific for the protein of interest), and was calculated as instructed by the manufacturer (www.universal.imaging.com). On average, calculations were performed after analysis of 50 different cells for four independent experiments.

Subcellular Fractionation and Immunoprecipitation Experiment—Cell fractionation was performed as described in Cartron et al. (20). Immunoprecipitation experiments were performed according to the manufacturer’s instructions (www.zymed.com/methods/ip.html). Bax or its mutants were incubated in the presence of $4 \mu g$ of antibodies 2D2, 6A7, Ab-4, TL41, or AF820 for up to 16 h at 4 °C. Antibody-protein complexes were then incubated with gentle rocking first with a second antibody for 2 h at 4 °C and then with Zsorbin (Zymed Laboratories Inc.) for an additional 2 h at 4 °C. The agarse beads were collected by centrifugation for 5 s at 13,000 × $g$. The supernatant was removed, and the pellet washed three times with phosphate-buffered saline before resuspension in 50 $\mu l$ of SDS-PAGE loading buffer. Controls immunoprecipitated (IP) were carried out in parallel with unrelated poly- or monoclonal antibodies. SDS-PAGE analysis and quantification of the IP IVT proteins using a PhosphorImager were performed as described for IVT protein insertion into mitochondria.

**Bacterial Two-hybrid Assay**—The interaction between Bax domains was assayed using the Bacteriometer$^{TM}$ two-hybrid system (Stratagene). This assay measured the interaction between polypeptides fused to the NT of RNA polymerase $\alpha$ in a target plasmid (pTRG) and a sequence fused to the bacteriophage $\lambda$cl protein in a bait plasmid (pBT). The interaction between the proteins ensured the survival of bacteria in a selective media. The binding of Bax $\alpha$1 with Bax BH3 was screened using as a bait the plasmid encoding for h-Bax $\alpha$1 (pBT- H$\alpha$1-BH3-RFP), whereas the target plasmids encoded for Bax $\alpha$ (pTRG-Bax $\alpha$), Bax $\psi$ (pTRG-Bax $\psi$), and Bax $\alpha$ deleted of its first 37 amino acids (pTRG-Bax A37), the BH3 region (pTRG-Bax A37H3), or expressing only this domain (pTRG-Bax BH3). Bacteria transformation, growth, and selection were performed according to the manufacturer’s instructions (www.stgm.com/manuals/982000.pdf).

**RESULTS**

The $\alpha$1 Mediates Bax Targeting to Mitochondria while the $\alpha$5a6 Is Involved in Its Membrane Insertion—It has recently been shown that the $\alpha$1 of Bax behaves as an endoplasmic reticulum transmembrane (TM) segment when fused to Escherichia coli Leader peptidase (25). In a previous study, we have shown that Bax $\alpha$1 is a mitochondrial targeting signal but not a membrane insertion domain for the MOM (26). Because, during apoptosis, Bax is inserted into MOM as a membrane-embedded protein (26), we have performed a series of mutants and deletions along the Bax $\alpha$ sequence to localize the anchor domain of Bax for the MOM. An epitope-mapping technique to detect changes in the conformation was also used to complement the mutagenesis experiments (Fig. 1 and Table II).

Cell free systems have been widely used to determine the nature of the signals contained in proteins that are involved in membrane integration, and indeed, this type of experiments have led to the demonstration that the CT of Bcl-2 contains a membrane addressing/anchoring signal (27, 28). Using the
involved in the membrane insertion of Bax.

We performed these mutations, which we et al. (10) have shown that the substitution of 8 out of 10 constructs were expressed in BdGBM cells (14), and apoptosis associated with mitochondria as observed in Fig. 2. This release was inhibited in hBax/L26G mutation abolished the association of Bax with mitochondria and this release was in hBax/L26G. These results suggest that hBax regulates the targeting, and the association of Bax with mitochondria and this release was inhibited in hBax/L26G.

TABLE II
Mapping of the conformation of the Bax constructs by immunoprecipitation with different anti-Bax antibodies

| Antibodies | 2D2 | 6A7 | AF820 | TL41 | Ab-4 |
|------------|-----|-----|-------|------|------|
| Amino acids Domains | 3–16 | 12–24 | 12–34 | 57–69 | 98–117 |
| Bax α | Wt + | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |
| Bax α h9a9 | L26G | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |
| Bax ψ | L26G | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |
| Bax ψ h9a9 | L26G | + | + | + | + |
|  | + | + | + | + | + |
|  | + | + | + | + | + |

The functionality of the association/insertion of Bax mutants was assessed by quantification of cyt c release into the supernatant in cell-free assay. As illustrated in Fig. 2b, only Bax ψ and Bax ψh9a9 induced the liberation of cyt c from mitochondria, and this release was inhibited in hBax/L26G. These results ruled out the possibility that Bax constructs, associated with mitochondria as observed in Fig. 2a, was simply aggregated on the surface of the organelle. These different constructs were expressed in BdGBM cells (14), and apoptosis was induced with DOX as described under “Experimental Procedures.” As shown in Fig. 2c, the deletion of hBax had no influence on the induction of cell death but increased the apoptogenicity of Bax ψ as described before (20). On the other hand, both the hBax and hBax5α6 mutations decreased cell death to that observed with a control plasmid (Fig. 2c). Altogether, these results suggest that hBax regulates the targeting, and the association of Bax with mitochondria and this release was inhibited in hBax/L26G.

Conformational changes induced by site-directed mutagenesis were analyzed by IP of Bax using different conformation-dependent or independent antibodies (Table II). The 2D2 antibody, which is directed against a NT peptide (amino acids 3–16) and cross-reacts with Bax α whatever its inactive/cytosolic or active/mitochondrial localization, and the anti-conformational antibody 6A7 directed against amino acids 12–24, which cross-reacts only with active/mitochondrial localized Bax (31), were used. Of note, the fact that Bax ψ reacts with the 6A7 antibody (Table II) suggests that the epitope recognized by this monoclonal antibody is present in the segment encompassing amino acids 20–24 in Bax α (i.e. 1–5 in Bax ψ sequence) contrary to what was reported by Hsu and Youle (32). We also used the antibodies AF820, TL41, and Ab-4 that are, respectively, directed against amino acids 12–34 (part of the ART domain and the hBax/L26G, hBax5α6, and hBax5α6 derivatives) had no effect on the association with mitochondria, and this release was inhibited in hBax/L26G.

Pro-13 and Pro-168 Participate in the Control of Bax Targeting and Insertion, Respectively—We have recently identified by site-directed mutagenesis that a Pro located at position 13 in the NT of Bax α was critical for the maintenance of the cytosolic conformation of Bax α (23). In our cell-free assay, the mutation of Pro-13 to Val (P13V), but not that of Pro8 (P8V), induced mitochondrial association and insertion of Bax α (Fig. S1a), as already reported (23). IP analysis revealed that the regions surrounding the hBax/L26G, hBax5α6, and hB3 region were exposed in Bax α P13V but not in Bax α and Bax α h9a9 (Table II). Note that the hBax/L26G, hBax5α6, hBax9 (Table II). These results suggest that Pro-13 is a key amino acid in the control of the association of Bax with mitochondria through conformational constraints. It has been proposed that two amino acids located in the CT of Bax α Pro-168 and Ser-184 played a similar essential role in Bax conformation (13, 21). Indeed a mutation of Ser-184 to Val (i.e. S184V) slightly increased the association of Bax α with mitochondria in a cell-free assay, whereas that of Pro-168 to Val (i.e. P168V) did not affect the association with mitochondria (Fig. S1b). However, in both cases, the insertion of Bax α into mitochondrial membranes was increased as judged by resistance to alkaline extraction (Fig. S1b). IP analyses revealed that the P168V and S184V mutations induced the externalization of hBax/L26G but not that of hBax (Fig. 3a). Despite the fact that the sequence preceding hBax/L26G has been remarkably conserved in all Bax orthologs, Pro-168 seems to play a particularly important role since a mutation of Thr-169 to Ala (i.e. T169A) had no effect on Bax α binding, its integration into mitochondria and/or a change in the conformation (Figs. S1a and 3a). These results suggest that Pro-168 and Ser-184 were not involved in the addressing of Bax to mitochondria but rather in a step promoting its membrane integration.
FIG. 2. Differential roles of hα1 and hα5α6 in Bax addressing and insertion in mitochondria. a, influence of the hα1 and the hα5α6, in the MOM targeting and insertion of Bax: Cell-free association and insertion of Bax variants (Bax α and Bax α with or without hα0) in which the hα1 (L26G) and/or the putative pore forming domain (hα5α6M) after incubated with purified rat liver mitochondria. Rat liver mitochondria were prepared as described under “Experimental Procedures.” IVT Bax constructs were added to 50 μg of mitochondria and incubated for 1 h at 37 °C. At the end of this reaction, the mitochondria were incubated in an alkaline-extraction buffer before SDS-PAGE/fluorographic analysis. Bax bound to mitochondria (Bound) or membrane integrated (i.e. resistant to the alkali treatment) (Inserted) were expressed as the percentage of the IVT Bax initially added to the reaction mixture (initial input). Results were obtained from at least four different experiments. b, influence of the hα1 and the hα5α6 in Bax-induced release of cyt c and DEV-Dase inducing activity from mitochondria in a cell-free assay: The cyt c released by the addition of the different Bax constructs to purified rat liver mitochondria was analyzed by immunobots in the supernatant of the cell-free assay mix. The measure of DEV-Dase activity induced in a non-apoptotic cellular extract by the same supernatant was performed as described under “Experimental Procedures.” Ct, DEV-Dase activity in non-apoptotic cellular extract in the presence (●) or in the absence (○) of total mitochondrial extract. The cyt c immunoblot shown is representative of the four different experiments, and the DEV-Dase data were calculated from four different experiments. c, influence of hα1 and hα5α6 mutations on in vitro induced apoptosis. BdGBM cells were transfected with the different Bax constructs and treated with DOX for 24 h before measuring the DEV-Dase activities in the cells. Similar amounts of Bax transgene were found in each situation. Cell death was determined by trypan blue exclusion and correlated to apoptosis as shown by DEV-Dase activities in the same cultures. Data shown are calculated from four different experiments. Ct, BdGBM cells transfected with the empty vector.

c. Subcellular localization of the constructs was calculated by determination of the correlation factor (CF) between the localization of the mitochondrial marker (i.e. MitoTracker Green™), cyt c, and that of the constructs, as described under “Experimental Procedures.” Of note equivalent expression of the different transgenes was found with the different Bax constructs (data not shown).

As illustrated in Fig. 3c, in the absence of an apoptotic stimulus, Bax α was found both in the cytosol and in the mitochondria of the cells (CF = 0.79), and, as expected, cyt c was only mitochondrial (CF = 1.0). Six hours after the induction of apoptosis by DOX, Bax α appeared to be mitochondrial (CF = 0.99), and cyt c was released from mitochondria and was present in the cytosol (CF = 0.69). Similarly, Bax α P13V was found to be predominantly mitochondrial (CF = 0.99) and generated a massive cyt c release (CF = 0.69), whereas the double mutant Bax P13V/L26G retained a cytosolic localization (CF = 0.77) and no release of cyt c was observed (CF = 0.99). Under the same conditions, the double mutant Bax α P13V/hα5α6M co-localized with mitochondrial (CF = 0.98) but did not generate a release of cyt c (CF = 1.0). On the other hand, Bax α P168V co-localized with mitochondria (CF = 0.97), and cyt c was partially released from mitochondria (CF = 0.72). The introduction of a mutation in the hα1, which inhibited the addressing of Bax to mitochondria (i.e. L26G) in the Bax α P168V

constructs exhibited an increased membrane insertion and this independent of the presence of hα0 (Fig. S1b). Quite importantly, these results showed that Pro-168 regulated the membrane insertion of Bax independently of its CT. To test the involvement of Pro-13 and Pro-168 in the interaction of Bax with mitochondria, P13G and P168V mutants that contained mutations in the addressing signal (L26G) or in the insertion domain (hα5α6M) were constructed. As illustrated in Fig. 3b, the association of Bax αP13V with mitochondria was impaired by the L26G mutation and its insertion inhibited with the hα5α6M construct. Mutations in hα5α6 inhibited the membrane insertion of Bax P168V. These data are in agreement with our previous observation that Pro-13 regulates the exposition of the hα5α6 region responsible for the insertion of Bax into mitochondria. This was further confirmed by IP analyses, which showed that hα1 and hα5α6 were exposed in the Bax P13V/L26G and Bax P13V/hα5α6 M mutants, whereas only the hα5α6 region was unmasked in Bax P168V/L26G and Bax P168V/hα5α6 M (Fig. 3c).

We then examined the activity and the intracellular localization of these constructs in a Bax (−) GBM (13). Confocal analyses allowed the determination of both the intracellular localization of these different constructs and that of cyt
mutant effectively inhibited Bax translocation to mitochondria (CF = 0.75) and no release of cyt c was observed (CF = 1). Conversely, the hα5α6 M mutation preserved the mitochondrial localization of Bax α P168V (CF = 0.98) but inhibited cyt c release (CF = 0.99). The DEVDase activities were enhanced in cells transfected with Bax α P13V and Bax α P168V compared with Bax α, whereas both the L26G and the hα5α6M in Bax, Bax P13V, and Bax P168V inhibited the induction of the caspase-like activity in DOX-treated cells (Fig. S2). These in vitro data confirmed the existence of a dual mitochondrial signaling in Bax, one responsible of mitochondrial addressing (i.e. hα1) and another for membrane integration (i.e. hα5α6).

The Role of an Electrostatic Bond between Asp-33 and Lys-64 in Bax Conformation—Recently, Lalle et al. (33) have shown that the hα1 of the anti-apoptotic protein Nr-13 interacted with its BH3 region possibly through electrostatic interactions. Because the 6A7 epitope has be found to be in the vicinity of the Bax dimerization domain (i.e. the BH3 region) (32), we tested the existence of an interaction similar to that of Nr-13, between Bax hα1 and its BH3 region. We used a two-hybrid assay in bacteria in which the one or more physical contacts between hα1 and the BH3 region were determined by the ability to produce a rescue phenotype upon interaction (see “Experimental Procedures”). Several Bax constructs with or without the BH3 region or hα1 were tested in this interaction assay using as bait pBT hα1-RFP, a construct in which Bax hα1 was fused to the cytosolic red fluorescent protein (RFP) (19). As shown in Fig. 4a, pTRG-(Bax Δ37) (Bax deleted of its first

**Fig. 3.** Evidence of the role played by Pro-13 and Pro-168 in Bax insertion into mitochondrial membranes. a, change of conformation induced by mutations as viewed by epitope mapping. In vitro synthesized [35S]Bax mutants were obtained and IP with various Bax antibodies with distinct epitopes (see Fig. 1) then analyzed on a SDS-PAGE and fluorography as described under “Experimental Procedures.” Wt, wild type (i.e. Bax a). b, influence of combined Pro-13 or Pro-168 mutations with hα1 and hα5α6 mutations on Bax α association with mitochondria: [35S]Bax constructs were incubated with rat liver mitochondria, and the association and insertion quantified as described in a. Data shown are the mean ± S.D. obtained from at least four independent experiments. c, subcellular localization of Bax Pro-13 and Pro-168 mutants. The mutated Bax proteins were expressed in Bax (−)/Bak (−) GBM cells, and their subcellular localization was analyzed before and after DOX-induced apoptosis. Laser confocal analyses were performed as described under “Experimental Procedures” using MitoTracker Green™ (Mito) to label mitochondria. Pictures illustrated are representative of at least three independent experiments. Magnification, ×60.
these residues in the association of Bax with mitochondria in a cell-free assay. In absence of t-Bid, the low binding of Bax to mitochondria in the cell-free assay was not affected by the mutations in hα1 and BH3 (Fig. 5a). On the other hand, the stimulation of Bax incorporation into mitochondria by p13-tBid was specifically increased in mutants of Lys-64 (i.e. K64A or K64D) or with L63D and inhibited by the D33A or D33K mutations (Fig. 5a). These results confirm that Asp-33 plays an important role in the activation of Bax by p13-tBid as already reported (34). IP of in vitro translated mutants indicated that the D33A and the K64A mutation induced a change in the conformation that could be detected with the AF 820 or the TL41 antibodies (Fig. 5b). The importance of these amino acids was further emphasized, because R34G or K21G mutants in hα1 and R65G or D68V mutants in BH3 did not affect the conformation of Bax, at least as estimated by IP (Fig. 5b). However, we cannot exclude that these amino acids do not play an ancillary but important role in the stability of the salt bridge (reviewed in Ref. 35).

The importance of a putative electrostatic interaction between Asp-33 and Lys-64 was confirmed in double mutants in which the charges were either inverted (D33K/K64D) or neutralized (D33A/K64A) the charges. As shown in Fig. 5b, the Bax α D33K/K64D double mutant retained the structure of Bax α, because it was immunoprecipitated only by the 2D2 antibody, whereas the D33A/K64A double mutant was recognized by both anti-hα1 and anti-BH3 antibodies (Fig. 5b). The association of the mutants with mitochondria in the cell-free assay showed that the double mutant Bax α D33K/K64D bound as poorly as Bax α, whereas the D33A/K64A mutation enhanced the binding of Bax α to mitochondria (Fig. 6). Of note, the Bax α D33A/K64A mutant was not inserted into mitochondrial membrane (data not shown). In agreement with the previous observation, Bax α D33A/K64A appeared to be inactive, because no release of cyt c and/or DEVDase-inducing factors was observed upon its binding to mitochondria (Fig. 6). These data suggested that the electrostatic interaction between the amino acids Asp-33 and Lys-64 is involved in the acquisition of a looser conformation of Bax, which is not per se sufficient to trigger the insertion of Bax in mitochondria. The comparison of the change of conformation of Bax induced by the P13V mutation with that induced by D33A implies that the exposure of the 6A7 epitope in addition of that of AF820 and TL41 appears to be correlated with the binding of Bax with mitochondria in the cell-free assay (compare Figs. 3a and 5b).

We also analyzed the influence of the expression of these mutants on the apoptotic response of Bax (−)Bak (−) GBM cells treated with DOX. Similar amounts of Bax α, Bax α D33A/K64A, or Bax α D33K/K64D were found in transfected cells suggesting that the mutations did not affect the expression and/or the stability of the protein (Fig. S3a). The intracellular localization of the mutants was analyzed by subcellular fractionation (Fig. 7a) and laser confocal microscopy (Fig. S3b). Both techniques showed that these mutations did not significantly modify the association of Bax in resting cells or upon the induction of apoptosis (Fig. 7a and Fig. S3b), contrary to what was observed in the cell free assay (Fig. 6a). The effect of these mutations on the induction of cell death by DOX was quantified by measuring cellular DEVDase activities and blue trypan exclusion (Fig. 7b). Compared with Bax α (i.e. Asp-33 through Lys-64), cells transfected with Bax containing mutations in the hα1 and in the BH3 region (D33A/K64A) exhibited less DEVDase activity upon DOX treatment (Fig. 7b). In addition, when the electrostatic bond between hα1 and BH3 was restored by the inversion of charges (i.e. D33K/K64D), the apoptotic activity of Bax was only partially recovered (Fig. 7b). A likely
explanation for the lack of activity detected with the latter mutant could be that mutation of Lys-64 enhanced the binding of Bax/H9251 to Bcl-2 (Fig. S4), and Asp-33 is central for the activation of Bax by proteases during apoptosis (36, 37).

Molecular Modeling of Domains Involved in the Control of Bax Addressing and Integration into Mitochondria—In resting cells, Bax is present in an inactive cytosolic conformation, which we called the cytosol locked in conformation (CLIC), whereas in apoptotic cells it acquires an active membrane-integrated conformation we called the cyt c liberation-associated conformation (CLAC). Bax/H9251 CLIC is a globular protein, which, upon apoptotic activation, undergoes changes in the conformation and several hidden domains become exposed in Bax/H9251 CLAC. A structural model can be proposed in which the NT and CT of Bax are, respectively, involved in the control of the addressing and the insertion of Bax into the MOM. One striking point of this model is that two prolines appear to be central in the control of the CLIC-CLAC conformation. Both residues are present in a solvent-exposed structure and near an α-helix (hα1 for Pro-13 and hα9 for Pro-168) (8), a structure favorable for a proline-driven conformational switch. Quite strikingly, a consensus sequence was found surrounding both Pro-13 and Pro-168 (i.e. GXXPT), which suggests that cis/trans changes in the conformation of the proline residues could be involved in the acquisition of the CLIC and CLAC conformations. This is confirmed by the mutagenesis studies, which show that a mutation of Pro-13 into a Val triggered Bax addressing to mitochondria whereas that of Pro-168 into a Val enhanced its membrane integration (Fig. 3). Although it is commonly associated with a helix breaker activity, prolines have also been involved in the control of the phosphorylation of preceding Thr/Ser residues. In the case of Pro-168, it has been suggested that this residue could be the target of prolyl-cis/trans-isomerase, which could be involved in the change in orientation of hα9 (21). However, in our hands, no experimental data support this hypothesis (data not shown). Note that because the NT of Bax is highly disordered (8), only the modeling of the kinks that could be produced by the GXXPT sequence in the CT of Bax is illustrated in Fig. 8a. Helical distortion caused by the isomerization of the proline could cause the exposure of hα1 in the

![Figure 5](http://www.jbc.org/)

**Fig. 5. Importance of amino acids Asp-33 and Lys-64 in Bax association to mitochondria in a cell free assay and conformation.**

a, effect of mutations of Asp-33 and Lys-64 on bax association with mitochondria. IVT Bax mutants were incubated with mitochondria as described under "Experimental Procedures." Addition of purified p13-tBid to IVT Bax was performed, prior to the addition to mitochondria, as described earlier (14). b, change of conformation induced by the D33A and K64A mutations. In vitro translated Bax mutants were IP with antibodies raised against different Bax epitopes and analyzed by SDS-PAGE and fluorography. Data shown are illustrative of three different experiments.
case of Pro-13 and that of hα5α6 in the case of Pro-168 and thus control the subsequent addressing and membrane insertion of Bax. In the case of the mitochondrial addressing of Bax, an additional step in the “disengagement” of the hα1 could be provided by the electrostatic bond between Asp-33 and Lys-64. As illustrated in Fig. 8b, the rupture of this salt bridge between the two amino acids was sufficient to provoke an opening in the Bax structure, which could free the hα1 and the BH3 region sufficiently, to allow its mitochondrial addressing (Fig. 6) but inhibit its activity in vitro (Fig. 7).

DISCUSSION

Structural changes involved in the activation of BCL-2 family members during apoptosis remain largely unknown (38). Numerous studies have shown that the CT of the anti-apoptotic proteins Bcl-2 or Bcl-xl is essential for its membrane insertion and anti-apoptotic function (reviewed in Ref. 21), although this view has been challenged in the case of Bcl-2 (39).

Several reports have shown that Bax undergoes a change in conformation upon the induction of apoptosis and that this step is necessary but not sufficient to facilitate its association and insertion into the MOM (1). The preservation of an inactive conformation of Bax has been suggested to be secured by the interaction between the NT and CT of Bax (13) but also by several proteins through an interaction with its NT and/or CT (i.e. Ku70, Humarin, e14-3-3) (40–42). Conversely, some of the BH3-only proteins such as Bid can induce the insertion of Bax into the MOM (1). Thus, from both in vivo and in vitro experiments, it appears that the control of a cytosolic conformation of Bax ensures the survival of the cells in the absence of an apoptogenic signal. These results suggest that the signal, which is responsible for the addressing of Bax to mitochondria, must be disclosed at an early stage of apoptosis. We and others have shown that the inactive form of Bax (i.e. Bax<sup>CLIC</sup>) interacts only weakly with mitochondria and that a deletion of the first 20 amino acids leads to a tight association of Bax (i.e. Bax<sup>CLAC</sup>) with mitochondria both in vitro and in a cell-free assay (12, 15, 23). The nature and the localization of the mitochondrial addressing and membrane-anchoring signals are still vividly debated. In several studies, the CT of Bax, as in
the case of Bcl-2 and Bcl-xl, was shown to be indispensable for its mitochondrial localization (reviewed in Ref. 26). However, it has been reported that a deletion of the CT of Bax does not always interfere with its apoptogenic activity or even with its mitochondrial localization 

in vitro

and/or in a cell-free assay (11, 15, 17, 20, 43, 44). Schinzel et al. (21) have observed that, upon the overexpression of Bax, the cells were killed by a mechanism that required the CT of Bax and that involved the translocation of Bax to the vicinity of mitochondria (but not to the outer membrane) where it formed a cluster with Bak. Remarkably, this structure appears to be similar to that observed during the late stages of apoptosis (45). In the same study, these authors observed that CT deleted forms of Bax aggregates in the cytosol, a feature that probably enabled these proteins to be translocated to mitochondria. It is thus possible that, under certain physiological situation (i.e. overexpression of Bax induced by p53) or in the late phase of

FIG. 7. Transfection of cells with hox1/BH3 mutants. a, subcellular localization of Bax α, D33A/K64A, and D33K/K64D. Cell fractionation was performed as described under “Experimental Procedures” on resting and DOX-treated cells for 24 h. b, effect of the induction of apoptosis of the expression of Bax α, Bax α D33A/K64A, and Bax α D33K/K64D transgenes in double deficient GBM cells. Cells were harvested 24 h after the induction of apoptosis by DOX, and apoptosis was quantified by measuring DEVDase activity and cell viability determined by trypan blue exclusion. (-), cell transfected with empty DNA vector. The data shown are the mean ± S.D. obtained from least three independent experiments.
apoptosis, the CT plays an important role in the formation of Bax/Bak clusters. Schinzel et al. (26) claimed that, providing the CT of Bax was extended to 23 amino acids, the CT led to an efficient mitochondrial targeting of cytosolic enhanced green fluorescent protein, but these authors did not show the importance of this “extra” residue in the targeting of full-length Bax to mitochondria. In the same report, it was shown that Pro-168 was a determinant in the targeting of Bax to mitochondria, and our results confirmed this observation. However, contrary to their results, we found that a substitution of this proline led to the translocation of Bax to mitochondria in mammals (Fig. 3). Quite remarkably, a consensus sequence GPTX...P, which introduced a reciprocal C-tail exchange model, accounting for the formation of Bcl-xl homodimers or heterodimers with Bax. These same authors also showed that the removal of the CT of Bax favors its heterodimerization with Bcl-xl, a feature that could also explain the inactivity of Bax ΔCT constructs in some cancer cell lines. We have observed a similar feature upon the co-expression of Bax and Bcl-xl in yeast (48). These latter results suggest that the CT of some members of the Bcl-2 family could fulfill different and complex functions, which remain to be clearly established.

On the other hand, because Hsu and Youle (49) have shown that residues Pro-13 through Ile-19 of the hox1 are hidden in the cytosolic form of Bax and become accessible to antibody binding after membrane insertion during apoptosis, the role of the NT has received increasing attention. This was emphasized by the observation that a sequence located in the NT of Bax called ART regulated the translocation of Bax to mitochondria (11). Indeed, we have recently observed that Bax α, an isoform of Bax α, deleted of the ART domain, was constitutively mitochondria-bound, and was highly apoptogenic (29). We have shown that the Pro-13 in Bax α plays an essential role in the mitochondrial localization of Bax and a substitution of this Pro-13 by a Gly (i.e. P13G mutation) induces Bax addressing to mitochondria and improves its pro-apoptotic activity (23). As illustrated in Table II, the hox1 was exposed in both Bax α and Bax P13G, and mutations in the hox1 such as L23G impairs both their association with mitochondria as detected in a cell-free assay and in vitro, as well as their apoptogenic activity, and this was independent of the CT of Bax. Mutations in the putative pore-forming helices hox5hox6 had no influence on the association of Bax with mitochondria in a cell-free assay and in vitro but inhibited its membrane integration and the subsequent release of cyt c (Fig. 2). These experiments showed that distinct domains were involved in the addressing and in the membrane integration of Bax. We further demonstrated that these two basic functions, often separated in nuclear encoded mitochondrial proteins (50), are under the control of two prolines (i.e. Pro-13 and Pro-168) located in the NT and in the CT of Bax (Fig. 3). Quite remarkably, a consensus sequence GXPT followed by a ho was found in both case to surround these prolines (8, 51). Similar sequences such as GXP, which intro-
duce a kink in α-helices, have been shown to be responsible for conformational switches that are important in the signaling of ion channels or G-coupled receptors (46). This structural inference as well as our experimental results and molecular simulations for Pro-168 (Fig. 8) could explain the role of these prolines in Bax function: both would be involved in the control of the disclosure of the addressing signal (Pro-13 controlling the inhibitory interaction of Bax NT with hα1) or that of the membrane integration domain (Pro-168 controlling the inhibitory interaction between hα9 and hα9α6). Modulation of these “proline switches” could be provided by physiological processes such as phosphorylation, modulation of the intracellular pH, or by direct interaction with regulatory proteins. An additional control of the exposure of the addressing signal, a major rate-limiting step in apoptosis, would be ensured by the interaction of hα1 with hα2, which contains the BH3 region (Figs. 6 and 7) and especially by the salt bridges formed by amino acids Asp-33 and Lys-64. The three-dimensional structure suggests that the BH3 region is not occluded by the NT as previously suggested and especially by the salt bridges formed by amino acids Asp-33 and Lys-64. The three-dimensional structure suggests that the BH3 region is not occluded by the NT as previously suggested and especially by the salt bridges formed by amino acids Asp-33 and Lys-64. The three-dimensional structure suggests that the BH3 region is not occluded by the NT as previously suggested

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Distinct Domains Control the Addressing and the Insertion of Bax into Mitochondria
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