The p18 Truncated Form of Bax Behaves Like a Bcl-2 Homology Domain 3-only Protein*

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The p21Bax is a pro-apoptotic member of the Bcl-2 family and is converted by calpain into a truncated form called p18Bax. This proteolysis enhanced the apoptogenic properties of Bax by a mechanism not yet elucidated. We have shown recently that the first a helix (H1) of p21Bax contained a mitochondrial addressing sequence, which appeared to be necessary for p21Bax-induced apoptosis (Cartron, P. F., Priault, M., Oliver, L., Meflah, K., Manon, S., and Vallette, F. M. (2003) J. Biol. Chem. 278, 11633–11641). This feature is in contradiction with the high apoptogenic profile of p18Bax, because the H1 is lost during the calpain cleavage of p21Bax. We investigated the role of p18Bax in apoptosis and found that its activity required the presence of p21Bax. In addition, p18Bax exhibited a higher affinity for Bcl-Xl than p21Bax did, a property that seems to be essential for the fulfillment of its pro-apoptotic role. In conclusion, calpain proteolysis converts the multi-domain p21Bax into a Bcl-2 homology 3-like protein capable of overcoming the inhibition of apoptosis due to Bcl-Xl.

Apoptosis is a cell death program that is central to cellular and tissue homeostasis and, as such, is involved in many normal and physiopathological processes (1). Proteins of the Bcl-2 family are the key regulators of this program, during which their main function is to control mitochondrial permeability and, in particular, the release of apoptogenic proteins from this organelle (2). Members of this family are divided into two main groups, the anti-apoptotic proteins with Bcl-2 and Bcl-Xl as archetypes and the pro-apoptotic proteins such as Bax. The pro-apoptotic proteins of this family can be subdivided into two groups. The first group is composed of Bax, Bak, and Bok and shares several domains of homology with Bcl-2 (i.e. Bcl-2 homology domains BH1, BH2, and BH3). The second group, typified by proteins like Bad or Bid, shared a homology with Bcl-2 limited to the BH3 domain and, hence, are called the “BH3-only proteins” (3). The ratio between these different proteins determines the sensitivity of the cell to apoptosis; anti-apoptotic proteins inhibit apoptosis by counteracting Bax and Bak, and BH3-only proteins either trigger apoptosis through direct interaction with Bax (i.e. Bid) or sensitize cells to death by inactivating Bcl-2 or Bcl-Xl (i.e. Bad) (4). The importance of the latter class of proteins has been emphasized by recent experiments using synthetic peptides, which have shown that the mere introduction of the BH3 domain could faithfully reproduce the death signals of the BH3-only proteins (3–9).

Members of the Bcl-2 family can form homo- and heterodimers among themselves or with other proteins (10). It is believed that this property constitutes the basis of the regulation of their functions and that the binding of Bcl-2 or Bcl-Xl to Bax or Bak and/or BH3-only proteins inhibits apoptosis, whereas the homodimerization of Bax or Bak promotes cell death (2, 10). The activity of the members of the Bcl-2 family is regulated in many ways; for example, the dephosphorylation of Bad allows its binding to the anti-apoptotic protein Bcl-Xl (11–14), and the cleavage of Bcl-Xl, Bid, or Bax by proteases (caspases, calpain, or cathepsin) enhances mitochondrial permeability (10). These results suggest that members of the Bcl-2 family can undergo post-translational modifications. This, in turn, allows these proteins to participate in the execution of apoptosis in addition to the complex inhibitory/activation interplay, which occurs at the onset of the apoptotic program (10).

In the case of Bax, several reports have shown that the cleavage of Bax by calpain or cathepsin D, at a late stage of apoptosis, removes the first 33 amino acids of the N-terminal, resulting in the transition from the p21Bax to the p18Bax form (15–18). However, the physiological significance of this cleavage is unclear and, in particular, it is not known whether p18Bax is important for the apoptotic mitochondrial function or merely a consequence of the activation of proteolysis in a late apoptotic phase. A recent report by Cao et al. (19) showed that a blockade of proteolysis by mutation of the calpain cleavage site inhibited the appearance of p18Bax and significantly delayed drug-induced apoptosis.

Goping et al. (20) have identified a domain called ART (apoptosis-regulating targeting sequence) in the first 20 amino acids of Bax that is important for the translocation of p21Bax to the mitochondria during apoptosis. Truncation of this domain enhanced the binding of Bax to mitochondria (20). We have recently reported that this deletion probably promoted the exposure of the H1 of Bax, which encompasses amino acids 20–37 and is a functional mitochondrial addressing signal necessary for p21Bax-induced apoptosis (21, 22). Because this sequence is lost in p18Bax, we have investigated its mechanism of induction of apoptosis in an attempt to address this apparent contradiction.

MATERIALS AND METHODS

Reagents—Unless otherwise stated, all reagents used in this study were from Sigma. The anti-N-terminal Bax antibodies 6A7 and 2D2...
were from R&D Systems, and the anti-C-terminal (i.e. amino acids 150 to 165) Bax antibody Ab-I was from Oncogene. Other commercial antibodies were used against actin from Chemicon (MAB1501R), anti-Bcl-2 from Dako (M0897), anti-Bcl-XL and anti-Bad from Transduction Laboratories (respectively B22630 and B36420), anti-Bid from R&D Systems (AF 846), and the anti-His tag from Qiagen (34660). The MitoTracker Green-FM was obtained from Molecular Probes.

**Bax Constructs**—p21Bax (Bax) and p18Bax (BaxΔ1–37) with or without the C terminus (169–192) were obtained by PCR amplification as described previously (21). The entire coding regions of p21Bax and p18Bax, cloned into pDEST 12.2 (Invitrogen), were subject to mutagenesis to obtain the L63E mutant using the site-directed mutagenesis system from Invitrogen with the oligonucleotide 5'-ACAAGAAAGAGTTCTTTTGGGAC-3' as the mutagenic primer (the mutated sequence is underlined). Similarly, the p21BaxD33A mutant (substitution of Asp-33 by Ala) was obtained by site-directed mutagenesis with 5'-TTTGGTACAGGTTCATCCCCAGCTCAGGGCCG-3' as the mutagenic primer (the mutated sequence is underlined).

**Cell Culture and Transfections**—Bax-deficient and Bax-expressing cell lines derived from human glioblastoma multiforme tumors (BdGBM and BeGBM, respectively) (23) were transfected with the empty vector (pDEST 12.2 from Invitrogen; control cells); Bax or the Bax mutants were subcloned into this vector (cf. above). Plasmid DNA (5 μg) was introduced into 105 BdGBM or BeGBM cells by electroporation (GenePulsar, Bio-Rad) using 200 V/cm and 250 farads. The transfected cells were selected in a medium containing neomycin (250 μg/ml) for 48 h.

**Cell-free Assay for Bax Insertion**—Mitochondria were prepared from normal rat liver, and the cell-free association of Bax with the mitochondria was performed as described previously (5, 21, 22). Briefly, [35S]Met (Amersham Biosciences) labeled proteins were synthesized from cDNAs using the TNT-coupled transcription/translation system from Promega. Post-translational insertion of labeled proteins into mitochondrial membranes was performed in a standard import buffer as described previously (21). [35S]Met-labeled proteins bound to the mitochondria were recovered in the pellet after centrifugation for 10 min at 4 °C at 8000 × g, and alkaline treatment of mitochondrial bound proteins was performed as described previously (31). SDS-PAGE of Bax-associated isolated mitochondria was analyzed in a SDS-PAGE and scanned with a PhosphorImager (Amersham Biosciences). The amount of protein present in the gel was subsequently quantified using the IP-Lab gel program (Signal Analytics, Vienna, VA). In some mitochondrial association experiments, [35S]Met-labeled Bax (4 fmol) and unlabeled Bcl-XL (4 fmol) were incubated together for 30 min before the addition of isolated rat liver mitochondria (50 μg) in the presence of Bax constructs (4 fmol) before the analysis of the insertion of [35S]Met-labeled Bax into mitochondrial membranes. The release of apoptogenic proteins from mitochondria was measured by the capacity of the mitochondrial supernatant to induce DEVDase activity in control cell extract (CCE) as described earlier (21). Protein binding experiments were performed as described (5). Briefly, the coding regions of Bcl-XL or Bcl-2 were subcloned into pDEST 17 (Invitrogen) to add a His tag at the N termini of the proteins. The His-tagged proteins were expressed in bacteria and purified according to the manufacturer’s instructions. In vitro His-tagged protein binding assays were performed as follows. p18Bax and p21Bax were in vitro translated and labeled with [35S]Met as described above. Equal amounts of each protein (4 fmol) were then diluted in 250 μl of binding buffer (142 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.4), 0.5 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors) and incubated with an equimolar concentration of the His-tagged Bcl-XL or Bcl-2 immobilized on nickel-Sepharose at 4 °C for 2 h. The resins were extensively washed with binding buffer, and then the His-tagged protein binding complexes were eluted with a buffer containing 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, and 250 mM imidazole. The presence of the labeled proteins was detected after an SDS-PAGE of the eluted fractions and the resin, and the radioactivity was quantified using the IP-Lab gel program.

**Quantification of Apoptosis**—The DEVDase activity in BDGMB cell extracts (2 × 106 cells) transfected with the different Bax constructs was measured as described previously (21, 22). Briefly, cell death was induced in Bcl-XL-BeGBM cells by a brief UV treatment (1 min) or by the addition of staurosporine (STS, 2 μM) and, in some cases, by a 10-fold excess of secondary antibody bound to agarose beads was added to the mixtures and incubated for 1 h at 4 °C. The lysates were then centrifuged for 2 min at 3000 × g at 4 °C, and the pellet was washed three times in Buffer B (Buffer A supplemented with 350 mM NaCl and 30% sucrose) and once in Buffer C (125 mM Tris-HCl (pH 6.8), and 0.1% SDS) before a final centrifugation for 5 min at 20000 × g at 4 °C. The pellet was resuspended in SDS-PAGE loading buffer. Western blots were performed as described earlier (21).

**Confocal Analysis**—For 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/aceton at 15 min at −20 °C. After saturation with 3% bovine serum albumin in phosphate-buffered saline, the cells were incubated with anti-Bax antibodies for 1 h at room temperature. After extensive washing, a secondary antibody coupled to Alexa 568 (Molecular Probes) was added overnight at 4 °C. Images were collected on a Leica TCS NT microscope with a 63 × 1.3 NA Fluotar objective.

**RESULTS**

**p18Bax Appearance and Its Role in Drug-induced Apoptosis in a Human Glioma Cell Line**—The cleavage of p21Bax into p18Bax by calpain and/or cathepsin during apoptosis has been reported in several tumor cell lines (15–19, 25, 26). We investigated the appearance of p18Bax in a human glioma cell line, BeGBM (23), treated with 2 μM STS to induce apoptosis. Cell death was monitored using the measurement of caspase-like activity (i.e. DEVDase) in cellular extracts (see “Materials and Methods”). As illustrated in Fig. 1A, the cleavage of p21Bax into p18Bax was associated with a marked increase in DEVDase activity at the beginning of the execution phase of apoptosis in BeGBM cells (23). Of note, similar results were obtained with UV-treated BeGBM (see below and Fig. 1C). To evaluate the importance of p21Bax cleavage during apoptosis, we produced a mutant p21Bax in which Asp-33, identified as the calpain cleavage site (15), was replaced by an Ala to give p21BaxD33A. This substitution has been shown recently to significantly inhibit apoptosis in several cell lines (15). We transfected a Bax-deficient human glioma cell line, BdGBM, with either p21Bax or p21BaxD33A and then analyzed STS- or UV-induced apoptosis and compared the results with that observed in BeGBM cells. As shown in Fig. 1B, the mere expression of p21Bax was not sufficient to trigger apoptosis in BdGBM cells as observed previously (22, 23). Similar results were obtained with p21BaxD33A. On the other hand, the expression of both p21Bax and p21BaxD33A increased the sensitivity of BdGBM cells to UV- and STS-induced apoptosis, although p21BaxD33A was not as efficient as p21Bax. As illustrated in Fig. 1C, similar amounts of p21Bax and p21BaxD33A were produced in BdGBM cells, and the D33A substitution inhibited the appearance of p18Bax. From these results, we concluded that the cleavage of p21Bax into p18Bax significantly enhanced apoptosis in GBM cells.

**p18Bax Efficiently Induced Apoptosis Only in the Presence of p21Bax**—To study the mechanism by which p18Bax induced apoptosis, we expressed transgenes encoding for p18Bax (see “Materials and Methods”) in BeGBM and BdGBM cells and compared the results with those obtained with similar p21Bax constructs. We used several constructs derived from p21Bax or

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P. F. Cartron, unpublished results.
p18\textsuperscript{Bax} in which the C terminus (i.e. amino acids 169–192) was deleted (ΔCT) or the Leu at position 63, critical for Bax homor heterodimerization (27), was replaced by a Glu to produce p21\textsuperscript{BaxL63E} and p18\textsuperscript{BaxL63E} (27). Clones expressing similar amount of p21\textsuperscript{Bax} and p18\textsuperscript{Bax} constructs were selected for this study (Fig. 2A). As reported by several authors (15–19, 25, 26), the expression of p18\textsuperscript{Bax} in p21\textsuperscript{Bax}-expressing cells induced apoptosis with an efficiency greater than that observed with
This property appeared to be linked to its capacity to dimerize, because the BH3 mutant (p18\text{BaxL63E}) was not active. It should be noted that a deletion of the C terminus (p18\text{BaxCT}) did not affect its function. Strikingly, no signs of cell death were recorded when p18 Bax was expressed in BdGBM cells (Fig. 2B). Of note, contrary to p21 Bax, which did not induce apoptosis per se in both cell types (Fig. 2B) but sensitized BdGBM cells to drug-induced apoptosis (Fig. 1B), the overexpression of p18\text{Bax} did not enhance the sensitivity of BdGBM cells to apoptosis (data not shown). These results suggested that p18\text{Bax} was not a direct inducer of apoptosis, as it appeared to require the presence of p21\text{Bax} to trigger cell death.

**p18\text{Bax} Induced the Translocation of p21 Bax to Mitochondria in Vitro**—The confocal data presented in Fig. 3A give more detailed information about the mechanism(s) involved in p18\text{Bax}-induced apoptosis. BeGBM cells were transfected with p18\text{Bax} His-tagged at its C terminus, because the latter segment does not influence p18\text{Bax}-induced apoptosis (Fig. 2). Anti-\text{His} antibodies were used to visualize p18\text{Bax}, whereas, to specifically detect p21\text{Bax} we used a Bax antibody (2D2) that recognized an epitope located in the N terminus of p21\text{Bax} (i.e. amino acids 3–16) that was not present in p18\text{Bax}. In BeGBM cells in the absence of p18\text{Bax}, the immunostaining observed with the 2D2 antibody indicated that the endogenous p21\text{Bax} was essentially cytosolic (Fig. 3A). Similarly, BdGBM cells transfected with p18\text{Bax} exhibited anti-\text{His} staining, which was essentially cytosolic (Fig. 3A). However, in BeGBM cells transfected with p18\text{Bax}, p21\text{Bax} appeared colocalized to the mitochondria with the 2D2 antibody, indicating that the expression of the p18\text{Bax} transgene induced the translocation of endogenous p21\text{Bax} from the cytosol to mitochondria (Fig. 3A). Of note, under these co-expression conditions, p18\text{Bax} also partially translocated to the mitochondria.

The antibody 6A7 is raised against an epitope located within the H\text{1} (amino acids 12–26), which becomes exposed when p21\text{Bax} is bound to mitochondria (28). As shown in Fig. 3B, when BeGBM cells were transfected with p18\text{Bax}, the p21\text{Bax} located in the mitochondria was labeled with the 6A7 antibody, suggesting that the p18\text{Bax} induced the translocation of p21\text{Bax} as well as the apoptogenic change in conformation. These results were in complete agreement with the p21\text{Bax}-dependent induction of apoptosis observed in Fig. 2.

**Association of p18\text{Bax} and p21\text{Bax} with Mitochondria in a Cell-free Assay**—The latter results led us to postulate that p18\text{Bax} behaved like a BH3-only protein, which induced apoptosis only through the multi-domain pro-apoptotic proteins Bax or Bak (6, 29, 30). One possibility was that p18\text{Bax} behaved like Bid and directly interacted with p21\text{Bax} to induce its apoptogenic...
change in conformation. This hypothesis was analyzed using an acellular assay (Fig. 4A). Briefly, in vitro translated 35S-labeled (IVT) proteins were incubated with purified rat liver mitochondria, and the binding of the different proteins to the organelle was quantified (Fig. 4B) as described under “Materials and Methods.” As shown in Fig. 4, IVT p21Bax (wild type) or p21BaxL63E (L63E) associated poorly with mitochondria, and this small membrane-associated fraction behaved as a peripheral bound protein, as it was completely removed with an alkaline treatment. Similarly, little or no association of p18Bax or p18BaxL63E with mitochondria was observed in the cell-free assay. Note that removal of the hydrophobic C termini of p18Bax and p21Bax (ΔCT) did not alter their poor pattern of association with mitochondria. The co-incubation of p21Bax with p18Bax did not enhance its binding to mitochondria, contrary to what was observed with the co-incubation of p21Bax with the BH3-only protein p13Bid (which is a truncated form of Bid) (Fig. 4). In strict contrast, the co-incubation of p18Bax with p13Bid did not influence the capacity of p18Bax to bind mitochondria (Fig. 4). These results argued against the fact that p18Bax directly activated p21Bax, at least in a p13Bid fashion. 

**p18Bax Exhibited a Higher Affinity for Bcl-Xl and Bcl-2 than p21Bax**

—BH3-only proteins can be categorized as either direct inducers of a change in the conformation of Bax that triggers its binding to mitochondria (e.g. Bid) or as inhibitors of anti-apoptotic proteins such as Bad (4) (e.g. Bcl-2). The previous experiments suggested that p18Bax could not behave like Bid. We have recently shown that a peptide derived from the BH3 domain of p21Bax was capable of promoting p21Bax insertion into the mitochondrial membrane in the presence of Bcl-Xl (5). We postulated that the capacity of p18Bax to bind Bcl-Xl or Bcl-2 could be involved in its p21Bax-induced apoptosis. The interaction of Bcl-Xl or Bcl-2 with p18Bax or p21Bax was studied in a cell-free assay using IVT 35S-labeled wild type, ΔCT, or L63E forms of p15Bax or p21Bax. The different IVT constructs were incubated with His-tagged Bcl-Xl or Bcl-2 immobilized on nickel-Sepharose, and proteins specifically eluted from the gel were analyzed and quantified as described under “Materials and Methods.” Of note, no residual IVT proteins were detected in the resins after the elution procedure (data not shown). As shown in Fig. 5, 35S-labeled p21Bax or p21BaxΔCT constructs bound to Bcl-Xl and Bcl-2 with a similar efficiency, and this binding was abolished by the L63E mutation. The IVT p18Bax and p18Bax ΔCT constructs bound to Bcl-Xl and Bcl-2 much more efficiently than p21Bax or p21BaxΔCT. This association was also abolished by the L63E mutation (Fig. 5), underlining the requirement of the BH3 domain of Bax in the heterodimerization of p18Bax with Bcl-Xl or Bcl-2.

**p18Bax-induced Apoptosis Is Mediated by Its Interaction with Bcl-Xl and p21Bax**

—The latter results suggested that p18Bax had a better affinity for Bcl-Xl than p21Bax had. To test the functional importance of this observation, BeGBM cells were stably transfected with Bcl-Xl to generate Bcl-Xl-BeGBM cells (Fig. 6A). Note that the expression of Bcl-Xl modified neither the expression of other key pro-apoptotic proteins (Bak, Bad, or p21Bax) nor that of the anti-apoptotic protein Bcl-2 (Fig. 6A). Bcl-Xl-BeGBM cells were more resistant to apoptosis induced by various cell death inducers (Fig. 6B), proving that, despite the fact that the transfection of these cells induced a relatively low overexpression of Bcl-Xl, the transgene was nonetheless functional. The p18Bax constructs (i.e. wild type, ΔCT, and L63E) as well as the BH3-only protein, Bad, were transiently expressed in Bcl-Xl-BeGBM cells. As illustrated in Fig. 6C, the expression of all the transgenes, except for that of
the L63E mutants, enhanced cell death in BeGBM cells. Quite spectacularly, the activation of caspases was even more important in Bcl-Xl-BeGBM as compared with BeGBM cells (Fig. 6C). Of note, p18Bax reversed the Bcl-Xl cytoprotective function more efficiently than the BH3-only protein, Bad, did (Fig. 6C).

We conclude that the presence of Bcl-Xl paradoxically enhances the p21Bax-dependent p18Bax-induced apoptosis.

To study further the interaction of Bcl-Xl with p21Bax and p18Bax in these cells, we analyzed the proteins co-immunoprecipitated with either the anti-Bax antibody (2D2) or the anti-Bcl-Xl antibody in BeGBM and Bcl-Xl-BeGBM cell extracts. As shown in Fig. 7A, in control BeGBM cells endogenous Bax (i.e. p21Bax) and Bcl-Xl co-immunoprecipitated, and this interaction was increased in Bcl-Xl-BeGBM cells. The expression of p18Bax in the latter cells completely abolished this interaction, whereas the expression of the p18BaxL63E mutant did not affect the p21Bax/Bcl-Xl complex (Fig. 7A). The direct interaction of p18Bax with Bcl-Xl was investigated in BeGBM and Bcl-Xl-BeGBM cells using His-tagged p18Bax. As illustrated in Fig. 7B, the p21Bax co-immunoprecipitated with the anti Bcl-Xl antibodies was replaced by the His-tagged p18Bax, which became associated with the pro-apoptotic Bcl-Xl. We also observed that p18Bax could successfully liberate a fraction of full-length Bid bound to Bcl-Xl (Fig. 7B). Because the mere expression of p18Bax was sufficient to induce apoptosis only in the presence of full-length Bax (see Fig. 2B), we hypothesized that the p21Bax released from Bcl-Xl, under these conditions, would thus be available for mitochondrial insertion.

Interaction of p21Bax, p18Bax, and Bcl-Xl in a Cell-free Assay—We further investigated the relationship between Bcl-Xl, p21Bax, and p18Bax, taking advantage of the cell-free assay described in Fig. 4. Purified rat liver mitochondria were incubated with IVT35S-labeled p21Bax in the presence or absence of IVT-unlabeled p18Bax, p18BaxL63E, or the BH3-only protein, Bad. As observed earlier, in the absence of Bcl-Xl, neither p18Bax, its mutants p18BaxL63E and p18BaxL63E, nor the BH3-only protein, Bad, enhanced the interaction of p21Bax with mitochondria (Fig. 8A). However, under the same conditions, p13tBid enhanced p21Bax binding and its subsequent integration into mitochondrial membranes (Fig. 8A). Similarly, in the presence of Bcl-Xl, both p18Bax and p18BaxL63E enhanced the binding of 35S-labeled p21Bax, detected as a membrane-embedded protein, to mitochondria as a result of its resistance to alkali treatment (Fig. 8A). Similar results were obtained with Bad (Fig. 8A). Note that the presence of Bcl-Xl and a functional p18Bax were required, as co-incubation of Bcl-Xl and p18BaxL63E with p21Bax did not trigger the insertion of p21Bax into mitochondrial membranes (Fig. 8A).

The association of p21Bax with mitochondria at the onset of apoptosis has been shown to be associated with the exposure of an epitope in the N terminus recognized by the monoclonal antibody 6A7 in vitro (28). On the other hand, the 2D2 antibody, which also recognizes an epitope present in the N terminus of p21Bax, does not exhibit such specificity and recognizes
both the soluble and the membrane-associated p21Bax (28). We immunoprecipitated p21Bax with either the 6A7 or 2D2 antibody in the presence of Bad or the different p18 Bax constructs and Bcl-Xl. As shown in Fig. 8B, in the absence of Bcl-XI, only the 2D2 antibody was capable of immunoprecipitating p21Bax. The latter result is in agreement with the fact that p21Bax, under these conditions, does not integrate with mitochondrial membranes. We use p13tBid as a positive control, as its interaction with p21Bax resulted in membrane integration (Fig. 8A) and exposure of the 6A7 epitope (Fig. 8B) in the absence of Bcl-XL. Conversely, Bad, p18Bax, and p18Bax/L63E, in the presence of Bcl-XI, induced a change in the conformation of p21Bax as detected by immunoprecipitation with the 6A7 antibody (Fig. 8B). Note that, in this experiment, the L63E mutation in p18Bax prevented the exposition of the 6A7 epitope in p21Bax (Fig. 8B).

Next, we investigated whether the membrane integration of p21Bax was accompanied by the induction of apoptotic-like mitochondrial permeability. The release of apoptogenic factors from the mitochondria was determined by quantifying the activation of caspase 3 in CCE by the supernatant obtained from treated rat liver mitochondria (see "Materials and Methods"). As shown in Fig. 8C, p21Bax incubated in the presence of mitochondria and the different p18Bax constructs did not release apoptogenic factors. In contrast, in the presence of Bcl-XI and p18Bax or p18Bax/L63E, the association of p21Bax with mitochondria promoted the liberation of apoptogenic proteins. This was determined by the capacity of supernatants from treated...
mitochondria to induce DEVDase activity in CCE (see "Materials and Methods"). Of note, in the presence of Bcl-XL and p18Bax, p21 Bax induced the release of such factors almost as efficiently as that produced by its co-incubation with p13 tBid (Fig. 8C). Conversely, p18 BaxL63E had no effect on mitochondrial permeability, whereas Bad behaved like p18 Bax and induced apoptogenic mitochondrial permeability in the presence of Bcl-XL (Fig. 8C).

DISCUSSION

The exact role of proteases other than caspases in apoptosis is still a pending issue. In this work, we investigated the molecular mechanism underlying the pro-apoptotic activity of a calpain-cleaved form of p21Bax, p18Bax. Previous results have shown that this truncated form was more apoptogenic than full-length p21Bax (15–19, 25, 26) and more resistant to the inhibitory properties of Bcl-XL (25). We have observed that, in the human glioma cell line, the cleavage of p21Bax into p18Bax occurred during the executive phase of apoptosis (i.e. after mitochondrial damage) and that inhibition of the generation of p18Bax, by modification of the p21Bax calpain cleavage site, resulted in a 40% reduction in cell death (Fig. 1). A similar result was observed by Cao et al. (19) during drug-induced apoptosis in leukemic cell lines. These experiments showed that, although it was neither essential nor necessary, p18Bax played an important role in the amplification of Bax-induced apoptosis. However, these results conflicted with our own re-

FIG. 7. p18Bax displaced p21Bax and Bid bound to Bcl-Xl in BeGBM cells. A, BeGBM (Cont.) or Bcl-Xl-BeGBM (Bcl-Xl) cells were transiently transfected with p18Bax and p18BaxL63E. Cellular extracts were immunoprecipitated (IP) with either the anti-Bcl-Xl or the 2D2 antibodies. The immunoprecipitated fractions were separated on SDS-PAGE, and immunoblots were analyzed for the presence of p21Bax (detected with the 2D2 antibody) or Bcl-Xl. Data illustrated are representative of three independent experiments. B, similarly as for panel A, BeGBM and Bcl-Xl-BeGBM cells were transiently transfected with His-tagged p18Bax. Cellular extracts were immunoprecipitated with anti-Bcl-Xl, 2D2, or anti-His antibodies as described under "Materials and Methods." The immunoprecipitated fractions were separated on SDS-PAGE, and immunoblots were probed for the presence of p18Bax (detected with the anti-His antibody), Bid, and p21Bax (detected with the 2D2 antibody). Data illustrated are representative of three independent experiments.

FIG. 8. p18Bax promotes the insertion of p21Bax in the presence of Bcl-Xl. A, integration of p21Bax into mitochondrial membranes in the presence of Bcl-Xl and the different p18Bax constructs in a cell-free assay. 35S-labeled p21Bax (4 fmol) was incubated with equimolar concentrations of unlabeled Bad or the different p18Bax constructs in the presence or in the absence of 4 fmol of unlabeled Bcl-Xl. Top, association of 35S-labeled p21Bax with mitochondria in the cell-free system. Bottom, membrane integration (fraction resistant to alkaline treatment) of 35S-labeled-p21Bax in the presence of the different constructs. The experiment illustrated is representative of three independent experiments. B, p18Bax promotes a change in the conformation of p21Bax in the presence of Bcl-Xl. Immunoprecipitation of p21Bax with the conformationally dependent monoclonal antibody 6A7 and the conformationally independent antibody 2D2 in the presence or absence of Bcl-Xl, Bad, or the different p18Bax constructs. The truncated form of Bid, p13 tBid, was added as a positive control for the induction of a change in conformation in Bax. The experiment illustrated is representative of three independent experiments. C, Bad and p18Bax promote a p21Bax-dependent release of apoptogenic factors from mitochondria in the presence of Bcl-Xl. The release of DEVDase inducing activity from mitochondria was assayed as described under "Material and Methods." The truncated form of Bid (p13-tBid) was added as positive control for the complete Bax-dependent release of apoptogenic factors. The experiment illustrated is representative of three independent experiments.
sults showing that the addressing signal was located within the sequence absent in p18Bax and that its removal led to the inhibition of Bax-induced apoptosis (22). We address this question by comparing the apoptotic potential of p18Bax in the presence or absence of p21Bax. A p18Bax transgene was expressed in glioma cell lines deficient or not in p21Bax (BdGBM and BeGBM) (23) and, as shown in Fig. 2, only BeGBM cells were sensitive to p18Bax. These results suggested that the p18Bax-induced apoptosis required the presence of p21Bax and, thus, that p18Bax could play only an auxiliary role during cell death. Indeed, p18Bax-induced apoptosis appeared to be specific for p21Bax because, although BdGBM cells expressed high amounts of Bak (29), the expression of p18Bax had no effect on apoptosis.

Interestingly, a mutation in the BH3 domain of p18Bax abolished its apoptogenic property and, thus, we hypothesized that p18Bax could behave like a BH3-only protein. BH3-only proteins require the presence of multi-domain pro-apoptotic proteins such as Bax and Bak (6, 29) and can be subdivided into “activators” of apoptosis, which trigger a p21Bax change in conformation (i.e. Bid), or into “sensitizers” of p21Bax-induced apoptosis, which successfully compete for p21Bax binding sites in Bcl-2 or Bcl-Xl (i.e. Badd) (4). Laser confocal analysis suggested that p18Bax induced the translocation of p21Bax to mitochondria (Fig. 3). However, as shown in Fig. 4, the addition of p18Bax, contrary to that of p13Badd, did not trigger p21Bax insertion into mitochondrial membrane in a cell-free system, eliminating p18Bax as a possible activator molecule. We have shown that the affinity of p18Bax for Bcl-Xl and Bcl-2 was higher than that of p21Bax (Fig. 5) and that, quite strikingly, the overexpression of Bcl-Xl enhanced the capacity of p18Bax to induce cell death (Fig. 6). Indeed, it has been reported in the literature that small organic compounds that mimic the BH3 domain or a peptide derived from the BH3 domain of Bax were more active in the presence of survival members of the Bcl-2 family (5, 31, 32). In the same line of thought, we have shown that, in vitro, p18Bax competes with p21Bax for binding with Bcl-Xl (Fig. 7). This observation is confirmed in the cell-free assay by showing that p21Bax, in the presence of p18Bax and Bcl-Xl, became membrane-inserted, which promoted the release of apoptogenic proteins from mitochondria (Fig. 8). We thus propose that the interaction of p18Bax with p21Bax-bound Bcl-Xl or Bcl-2 led to the liberation of an active pro-apoptotic p21Bax. This could be a general feature of the BH3 domain of Bax because, as shown in Figs. 6 and 8, the pro-apoptotic “sensitizer” Bad shared this property, at least partially. The direct interaction between Bcl-2 or Bcl-Xl and Bak or Bax is still an unresolved issue. Originally it was proposed that Bak was inactivated through its binding to Bcl-2 (33), but this view has been challenged by several reports that suggest that this type of heterodimerization did not occur under normal physiological conditions (34). On the other hand, this interaction has been reported in the literature, because the Bcl-2 viral homologue E1B (19 kDa) binds to “open conformers” of Bak (35), and interaction between Bax and Bcl-2 has been shown by direct measurement using fluorescence resonance energy transfer (36). It is possible that Bcl-2 or Bcl-Xl neutralize partners involved in Bax or Bak activation, thus preventing their deleterious effects (6). The BH3-only protein Bid could provide this missing link, as its binding to Bcl-2 and/or Bcl-Xl would prevent its activation of Bak and/or Bax. Interestingly, in this work we show that both Bax and Bid can be immunoprecipitated with Bcl-Xl in vitro (Fig. 7).

From our results, a model can be envisaged in which, during apoptosis, p13Badd induced a change in conformation in p21Bax that led to its activated form and, thus, promoted a change in the permeability of the mitochondrial membranes. The latter change provoked the activation of proteases such as calpain, which, in turn, converted p21Bax into p18Bax. On the other hand, p21Bax could be inactivated through its interaction with Bcl-2 or Bcl-Xl. If this interaction was direct, it is likely to occur through the BH3 domain and, thus, would result in the disruption of a hydrophobic groove in which this domain is hidden in the inactive p18Bax (37). Thus, the formation of an inhibitory complex would lead to a more “open” conformation of p21Bax (i.e. exposition of the BH3 domain of Bax) although not to a fully active state, as an interaction of p21Bax with Bcl-Xl does not induce the appearance of the 6A7 epitope (5). From this complex, p18Bax would finally remodel the p21Bax toward an active 6A7 positive form and, thus, liberate a fully active p21Bax. The precise mechanism by which this activation could be achieved remains to be established.

In conclusion, in this work, we report that p18Bax did not induce apoptosis in the absence of p21Bax and did not associate with mitochondria. This result is in agreement with our previous report that shows that the addressing/targeting mitochondrial sequence of p21Bax is present in the region encompassing the Ha1 (21) which is absent in p18Bax. During apoptosis, p18Bax could ensure the completion of the cell death program by overcoming the inhibition by Bcl-Xl or Bcl-2, thus providing a necessary “coup de grace” for the cells. Interestingly, Gillissen et al. (38) have recently shown that the BH3-only protein Nbk/Bik induced apoptosis only in the presence of Bax, although it did not interact directly with it. The latter result, which is in perfect agreement with our findings concerning p18Bax, suggests that BH3-only proteins can be further subdivided into Bax-dependent and Bax-independent activators of apoptosis and provides the basis of a new modus operandi for the BH3-only proteins. In addition, the efficacy of p18Bax to induce apoptosis in Bcl-2-overexpressing cells is relevant to cancer therapy, because overexpression of anti-apoptotic members of the Bcl-2 family is frequent in tumors (39), and Bcl-Xl has been correlated to tumoral resistance to chemotherapy (40). Thus, one can speculate that p18Bax would specifically induce apoptosis in vitro in the most aggressive tumoral cells.

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