The regulated oligomerization of proteins is increasingly understood to be an important step in many cellular processes, including signaling, transcription, and protein degradation. The activity of Bax, which is essential for the completion of apoptosis, has been shown to be associated with its oligomerization: homodimerization that appears to facilitate mitochondrial permeabilization during apoptosis and heterodimerization with multidomain anti-apoptotic members of the Bcl-2 family inhibiting this process. Several domains have been identified to be crucial in the homo-/heterodimerization or oligomerization of Bax, especially the so-called Bax homology 3 domain. In this study we show that although the carboxyl terminus of Bax is not implicated in its mitochondrial localization, it has a role in the dimerization process and thus in its activity.

Proteins of the Bcl-2 family (pBcl-2s)² are essential for the implementation of the cell death program called apoptosis (1). This family is defined by structural and sequence homology of at least one domain. Typically, anti-apoptotic members of this family (Bcl-2, Bcl-xL, and Bcl-W) comprise four domains of homology termed BH1–4, whereas pro-apoptotic members are limited to three domains BH1–3 (multidomain proteins such as Bax and Bak) or only a BH3 (e.g. Bad or Bid) (1). The latter class is divided into two subgroups, Bad-like proteins that act as inhibitors of anti-apoptotic members or Bid-like proteins that act as activators of Bax (2). The BH3 domain is implicated in the homo- and heterodimerization of pBcl-2s, a process that is central to the control of mitochondrial permeabilization, which is essential for the completion of the apoptotic program (3). The apoptotic mitochondrial outer membrane permeabilization (AMOMP), which leads to the release of intermembrane mitochondrial proteins such as cytochrome c, Smac/Diablo, and AIF, is under the control of Bax and Bak (3). In healthy cells, Bax is a monomeric protein soluble in the cytosol or loosely attached to the outer mitochondrial membrane. Upon the induction of apoptosis, Bax undergoes a change in the conformation resulting in the translocation to the mitochondria and the subsequent formation of oligomers, which are supposed to be involved in the formation of AMOMP via the formation of channels or pores (4). Thus, oligomerization appears to be essential to AMOMP, but the precise molecular mechanism is not completely understood. Recent data from Annis et al. (5) suggest that membrane permeabilization occurs through the recruitment of monomers embedded in the membrane, and thus the assemblage of oligomers occurs within the mitochondrial membrane or in close proximity and not in the cytosolic compartment. It has been suggested that the last α-helix of Bax (i.e. Hα-9) was the addressing/membrane anchor domain of the protein based on its homology with equivalent domains in anti-apoptotic pBcl-2s (6). However, we and others have shown that this domain was not essential for either its mitochondrial addressing or for its pro-apoptotic activity in yeast and mammals (reviewed in Ref. 4). In addition, Bax lacking its Hα-9 (BaxΔC) appears to be more effective than full-length Bax α (FLBax) to form channels in the mitochondrial membrane (7). Structural analyses suggest that although the Hα-9 of Bax is anchored in the membrane, its actual role is more complex than primarily assumed (4).

In this work, we have analyzed apoptosis induced by a Bax construct lacking the Hα-9 (BaxΔC). We show that BaxΔC induced apoptosis in a cell line deficient in endogenous Bax as well as in a cell line expressing Bax. Interestingly, the BaxΔC activity seems to depend on the presence of endogenous Bak. However, BaxΔC appears to be a monomer both in the cytosol and in the mitochondria under healthy or apoptotic conditions suggesting that BaxΔC cannot form oligomers with itself or with FLBax. We also show that substitution of Hα-9 by the equivalent segment of Bcl-xL (BaxTMxL) restores this capacity to form oligomers in a cell-free assay, thus confirming that the Bax-CT is not involved in Bax oligomerization.

**EXPERIMENTAL PROCEDURES**

*Materials—Unless otherwise stated, all reagents used in this study were from Invitrogen. Etoposide was from Teva® Classics (Paris, France) and was used from a stock solution at 20 mg/ml. Commercial antibodies used were monoclonal anti-Bax (clone 2D2, R & D Systems, Lille, France), polyclonal anti-Bax (clone A33533, DakoCytomation, Trappes, France), monoclonal anti-FLAG (F1804, Sigma), monoclonal anti-cytochrome c (MAB-897, R & D Systems), horseradish peroxidase-coupled secondary antibodies from Bio-Rad, and Alexa 568- and 488-conjugated sec-

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and S2.

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2 The abbreviations used are: pBcl-2s, proteins of the Bcl-2 family; BH, Bax homology; BN-PAGE, blue native PAGE; AMOMP, apoptotic mitochondrial outer membrane permeabilization; Hα, α-helix; Bax-CT, carboxyl terminus of Bax (amino acids 170–193); FLBax, full-length Bax α; GFP, green fluorescent protein; IVT, in vitro translated; NT-Bax, amino terminus of Bax; RFP, red fluorescent protein; rt-Bid, recombinant truncated (p13)-Bid; PBS, phosphate-buffered saline; EGS, ethylene glycol bis(succinimidy1 succinate); siRNA, small interfering RNA; DSS, disuccinimidyl suberate.
ondary antibodies from Molecular Probes (Invitrogen). The cross-linkers ethylene glycol bis(succinimidy1 succinate) (EGS) and disuccinimidyl suberate (DSS) were from Pierce.

Cell Culture—The human breast carcinoma cell line HeLa was grown in complete RPMI (RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate). The human prostate carcinoma cell line Du145 (gift from Dr. P.T. Daniel, Max Delbruck Center for Molecular Medicine, Berlin-Buch, Germany) was grown in complete Dulbecco’s modified Eagle’s medium (Dulbecco’s modified Eagle’s medium, 4500 mg/liter glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate). Cells were cultured at 37 °C in an environment of 5% CO2, 95% air in a humidified atmosphere. Apoptosis was induced in semi-confluent (50–70%) cultures, and cell viability was determined by trypan blue exclusion.

Plasmid Constructions—pRcCMV/FLBax, pRcCMV/BaxΔC, and pRcCMV/BaxTmXL were as described previously (8). GFP-FLBax and GFP-BaxΔC were obtained by site-directed mutagenesis using the PCR-based Gateway method and then subcloned into a pDEST12.2 plasmid according to the manufacturer’s instructions (Invitrogen). Primers used were FLAG sense (5′-GGG GAC AAC TTT GTA CAA AAA AGC AGG CTT TAT GGA CGG TGC CGG GGA GCA GCC C-3′) and Bax antisense (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GCC CAT CTT CCT CCA GAT GGT G-3′) (for Bax) or BaxΔC antisense (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GCC CAT CTT CCT CCA GAT GGT G-3′) (for Bax ΔC) subcloned into a pDEST12 plasmid, and confocal analysis using the same photomultiplier setting was performed.

Bax Dimerization—FLAG-FLBax, cloned into pDEST12.2, was subjected to mutagenesis to obtain the L63E mutant using the site-directed mutagenesis system from Invitrogen with the oligonucleotide 5′-ACC AAG AAG CTG AGC GAG TGT GAG AAG CGC ATC GGG GAC-3′ (the mutated sequence is underlined). The construction of the Hα1-RFP and RFP-Ho9 plasmids was as described previously (9).

Cell Transfections—The different cell lines were transfected with pRcCMV-FLBax, pRcCMV/BaxΔC, or pDEST12.2-FLAG-BaxΔC using Lipofectamine™ 2000 (Invitrogen). Briefly, 10 μg of plasmid DNA diluted in 100 μl of Opti-MEM® medium was added to 25 μl of Lipofectamine, gently mixed and incubated for 15 min at room temperature, and then added directly to the cells at 50–70% confluency. Stable transfectants were selected with 250 μg/ml G418. For HeLa cells transfected with GFP-Bax (pcDNA-DEST53) or GFP-BaxΔC (pcDNA-DEST53), 10 μg of plasmid DNA was introduced by electroporation (GenePulser, Bio-Rad) using 200 V/cm and 250 microfarads, and transfected cells were selected as above. All cells were used as stable transfectants.

Cell Fractionation—After the induction of apoptosis by UV irradiation (1 min) or etoposide treatment, the cells were collected (16, 24, or 36 h after treatment) and centrifuged at 1,000 × g for 10 min at 4 °C. The cell pellets were washed twice with ice-cold PBS 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 mM cytochalasin B, 1 mM EGTA, and 1 mM protease inhibitor), as described previously (1). Cells were allowed to swell for 30 min on ice and then homogenized with 30 strokes in a 2-ml glass Dounce homogenizer. The homogenates were centrifuged at 750 × g for 10 min at 4 °C, and the resulting supernatants were centrifuged a further 15 min at 15,000 × g at 4 °C. These mitochondrial pellets were resuspended in CEB. The supernatants were further centrifuged at 100,000 × g for 30 min in an Airfuge (Beckman Instruments), and the resulting supernatants (S100) were further referred to as the cytosolic fraction.

Microinjection—Microinjections were performed as described previously (11). Briefly, cells were seeded on glass coverslips the day prior to microinjection, which was performed using sterile micropipettes (femtotips II, Eppendorf, Hamburg, Germany) mounted on an automated microinjection system. The plasmids (100 ng/μl) were co-injected with 100 ng/μl pDSRed2-C1 plasmid marker (Clontech). Typically, 100–200 cells were injected using an identical pressure (100 hPa) and time (0.1 s). The percentage of positive cells (with red fluorescence) with apoptotic characteristics was evaluated every 2 h. For cytoplasmic microinjections, peptides and/or recombinant proteins were dissolved in PBS together with dextran (10 kDa)-conjugated lysine-fixable Oregon Green (final concentration of 0.5%; Molecular Probes) as a co-injection marker. Typically, 200 cells were microinjected for each condition in each experiment. Calibration assays indicated that a 2- to 5-pl volume was delivered to each cell (data not shown). The percentage of positive (i.e. fluorescent) cells exhibiting morphological features of apoptosis was evaluated by fluorescence microscopy.

For nuclear microinjection studies, plasmid DNA was purified using Qiagen midipreps according to the manufacturer’s instructions. 100 ng/μl empty expression vector pEGFP-N1, pEGRF-N1 (FLBax), or pEGFP-N1 (BaxΔC) was microinjected. The cells were microinjected with the same concentration of plasmid, and confocal analysis using the same photomultiplier transmission confirmed that the relative fluorescence in the cells microinjected with FLBax and BaxΔC was similar (data not shown).

Cross-linking Experiments—For cross-linking experiments with EGS or DSS, 50 μg of mitochondrial or cytosolic fractions (S100) were incubated in the absence or in the presence of 2 mM EGS or DSS for 45 min at room temperature. Alternatively, the cross-linker DSS was added to IVT-FLBax, BaxΔC, Hα1-RFP, and RFP-Ho-9 at a final concentration of 5 mM at pH 7.8 after triggering dimerization (10). After incubation for 30 min at room temperature, the cross-linker was quenched with 25 mM Tris-HCl (pH 7.5). Bax oligomerization was analyzed in 12% SDS-PAGE, followed by PhosphorImager analysis.
Bax Dimerization

**A**

Western Blots—The protein concentration was determined in the different fractions using the Bio-Rad protein assay prior to analysis under reducing conditions in 12% SDS-PAGE. Western blots were performed using standard protocol. BM chemiluminescence blotting substrate peroxidase (Roche Applied Science) was used to detect proteins bound to Immobilon-P (Millipore).

**B**

**Laser Confocal Microscopy**—Confocal analyses were performed as described previously (11), and the cells were fixed with 4% paraformaldehyde, 0.9% picric acid in PBS for 30 min. After a 10-min permeabilization with 0.1% SDS/PBS and a saturation with 5% gelatin/PBS for 1 h, the cells were incubated with polyclonal anti-F1-ATPase (1/1000; a gift from Prof. J. Lunardi) for 1 h and then washed and incubated overnight at 4 °C with anti-Bax antibodies (2 μg). After extensive washing, the cells were incubated with secondary antibodies for 1 h. All incubations were done at room temperature unless stated otherwise. Images were visualized using a Leica TCS NT microscope with a ×63 1.3 NA Fluar objective (Leica, France). Quantification of the overlay of labeling was done using the Metamorph program. Briefly, the correlation coefficient ($r$) is a measure of the strength of the relationship between two variables $x$ (labeling specific for mitochondria) and $y$ (labeling of Bax).

**Blue Native-PAGE**—Blue native-PAGE (BN-PAGE) was performed essentially as described on line. Briefly, mitochondria were suspended at 0.8 mg/ml in imidazole/HCl buffer (pH 7.0) containing 50 mM NaCl and 5 mM e-aminocaproic acid. Digitonin solubilization was done for 20 min on ice at a detergent/protein ratio of 3 (w/w). Unsolubilized material was removed by centrifugation at 21,000 × g for 20 min. The supernatant was supplemented with Coomassie blue G in 750 mM e-aminocaproic acid at a dye/protein ratio of 0.2 (w/w) and separated on a linear gradient (5–14%) polyacrylamide slab gel. Molecular weight markers were treated in a similar manner before loading on the gel. Second dimension electrophoresis was done on each individual lane excised from the BN-PAGE. The excised lanes were incubated in dissociating solution (1% SDS, 1% β-mercaptoethanol) for 3 h at room temperature. The lane was layered on the top of a 12.5% SDS-PAGE. The proteins were analyzed as described above.

**Quantification of Immunoblots—**Bands were quantified using the Image J program available on line.

**Induction of Oligomerization**—[35S]Met proteins were synthesized from cDNAs using the TNT-coupled transcription/translation system from Promega (Lyon, France). The oligomerization of IVT-Bax, FLAG-Bax, BaxΔC, FLAG-BaxΔC, Bax-L63E, or BaxTMxL was induced by either recombinant truncated (rt)-Bid (11), increasing the temperature from 30 to 43 °C for 1 h (12), or changing the pH (10). rt-Bid was eliminated by nickel-nitrilotriacetic acid (Qiagen) as described previously (11). Low binding Ultracl YM membrane from Millipore (France) with a cut off of 50 kDa (MicroCon YM 50) was used to isolate IVT-Bax monomers (21 kDa) or dimers (42 kDa), as recommended by the manufacturer. IVT proteins incubated at various conditions (temperature, pH, or tBid) were added to microCon YM 50 and were then eluted from the column and verified by SDS-PAGE and autoradiography as described previously (10).

**Immunoprecipitation**—Immunoprecipitation experiments were performed using Catch and Release version 2.0 (Chemicon, Millipore, France) under native condition with IVT-Bax or its mutants activated by 8 fmol of rt-Bid or by raising the temperature from 30 to 43 °C for 1 h and with 4 μg of anti-FLAG antibodies according to the manufacturer's instructions. Antibody-protein complexes were then incubated with gentle rocking for 30 min. The resin was collected by centrifugation for 30 s at 2,000 × g. The supernatant was removed, and the pellets were washed, and then the proteins were eluted with 1 × non-denaturing elution buffer by centrifugation for 30 s at 2,000 × g. Control immunoprecipitations were carried out in parallel with unrelated poly- or monoclonal antibodies. Immunoprecipi-
tated proteins were analyzed by SDS-PAGE and scanned with a PhosphorImager (GE Healthcare).

Knockdown of the Expression of Bak in Du145 Cell Line—The expression of Bak was knocked down in Du145 cell lines by the Silencer/H23041 siRNA construction kit (Ambion) as described on line. Three different siRNA sequences were used: siRNA Bak137 sense, 5′-GAT CCG CAG GAG GCT GAA GGG GTG GTT CAA GAG A CCA CCC CTT CAG CCT CTT GT TTG GAA A-3′, and antisense, 5′-AGC TTT TCC AAA AAA TGG TCC CAT CCT GAA CGT GTC TCT TGA ACC ACC CCT TCA GCC TCC TGCC-3′; siRNA Bak316 sense, 5′-GAT CCG TGC CTA TGA GTA CTT CAC CTT CAA GAG A GGT GAA GTA CTG ATA GGC A TTT TTT GGA AA-3′, and antisense, 5′-AGC TTT TCC AAA AAA TGC CTA TGA GTA CTT CAC CTC TCT TGA AGG TGA AGT ACT CAT AGG CAC G-3′; siRNA Bak553 sense, 5′-GAT CCG TGG TCC CAT CCT GAA CGT GTT CAA GAG A CAC GTT CAG GAT GGG ACC A TTT TTT GGA AA-3′, and antisense, 5′-AGC TTT TCC AAA AAA TGG TCC CAT CCT GAA CGT GTC TCT TGA ACA CGT TCA GGA TGG GAC CAC G-3′.

RESULTS

A Deletion of the Bax-CT Does Not Interfere with Its Intracellular Localization and Its Apoptotic Function but with Its Oligomerization in a Bax-deficient Cell Line—We and others have shown that the Bax-CT was not essential for its subcellular localization or its apoptotic function in several cell lines (8, 9, 14–16). The prostate carcinoma cell line Du145, which does not express Bax, was transfected with a human Bax construct in which the last 21 amino acids (i.e. amino acids 170–191) were deleted, and a FLAG tag was added to the amino terminus (FLAG-BaxΔC). The subcellular localization and apoptotic activity of this construct was compared with that of full-length Bax α (FLBax). It should be noted that we were not able to
obtain a stable transfected cell line exhibiting a high expression of Bax/H9004C as the overexpression of this construct appeared highly toxic for these cells (data not shown). As established in Fig. 1A, the cell lines used exhibited a 2-fold difference between FLBax and FLAG-Bax/H9004C variants. Interestingly, despite this difference in expression, the viability of cells was more affected in the Bax/H9004C-expressing cells (Fig. 1B), suggesting again that the construct was highly toxic. Of note, under our conditions, time-lapse experiments showed that the kinetics of cell death of the FLBax- and Bax/H9004C-Du145 cells were similar (supplemental Fig. 1).

One of the hallmarks of apoptosis is the relocalization of Bax from the cytosol to mitochondria (17). To determine whether the release of cytochrome c was associated with a Bax translocation to mitochondria, the subcellular localization of Bax and BaxΔC in untreated and etoposide-treated cells was analyzed by laser confocal microscopy. As shown in Fig. 2A, a proportion of Bax and BaxΔC was mitochondrial (i.e. co-localized with F1-ATPase) in untreated cells, and this proportion was significantly increased in both cases after the induction of apoptosis by the DNA-damaging agent etoposide. Similar results were obtained using another apoptosis inducer (i.e. UV-B treatment) (data not shown). To verify that the cytochrome c release was associated with Bax (and in particular that of FLAG-BaxΔC) translocation to mitochondria, its presence in the cytosol was assessed after cell fractionation as described earlier (9) in both FLBax and FLAG-BaxΔC-expressing Du145 cells. As shown in Fig. 2B, induction of apoptosis by etoposide stimulated the liberation of cytochrome c in both FLBax- and BaxΔC-transfected Du145 cells, although in the latter case this release was not complete. The extent of the release of cytochrome c has been correlated with the degree of oligomerization of Bax (18–20). Thus, we examined the molecular organization of Bax and BaxΔC after the induction of apoptosis by UV-B or etoposide. Fig. 2C shows the results obtained in UV-B-treated cells (note that similar results were obtained with etoposide). Quite surprisingly, we observed that the oligomerization of Bax was incomplete in mitochondria from FLBax-transfected Du145 cells in which only dimers were found and completely absent in BaxΔC transfected Du145 cells in which only monomers were observed.

These results suggest that the oligomerization of Bax is affected in Du145 cell lines, and this is particularly significant in the case of BaxΔC as this variant is present only as monomers. It has been suggested that c-Myc was involved in Bax activation at the mitochondrial level and, in particular, in promoting the
oligomerization (5, 21). However, it has been reported that Du145 cells express a functional c-myc (22), ruling out the involvement of this oncogene as a putative factor involved in the deficient oligomerization.

The putative role of Bak in Bax activity has been raised by several reports (1–5), and one possibility was that Bax/H9004C activated the apoptotic program through heterodimerization with Bak. Because we observed only the monomeric form of Bax/H9004C, we investigated a possible physical association with Bak using co-immunoprecipitation experiments. Fig. 3A shows that Bax did not co-immunoprecipitate with Bak under any of our conditions. We expressed the Bax/H9004C construct into Du145 cell lines treated with several siRNAs designed to knock down the expression of Bak (see “Experimental Procedures” and Fig. 3B) to study the influence of endogenous Bak on Bax/H9004C activity. Apoptosis was induced either by UV irradiation or by staurosporine treatment in co-transfected cells, which expressed similar amounts of Bax/H9004C (data not shown) and different amounts of Bak (Fig. 3B). Cell death was monitored by trypan blue exclusion as described earlier (11). In the Du145 cell line, which expressed Bak but not Bax, cell death was accompanied by oligomerization of Bak (supplemental Fig. 2). This result suggests that the absence of Bax did not interfere with Bak activity. In contrast, little or no cell death was observed in cell lines devoid of Bak indicating that the presence of Bak affects the activity of Bax/H9004C (Fig. 3C). Of note, similar results were obtained with full-length Bax (data not shown), indicating that the role of Bak in Bax activity is not dependent on the presence of Bax-CT.

The Oligomerization of Bax/H9004C Is Also Absent in Bax-expressing HeLa Cells—it could be possible that the lack of endogenous FLBax was responsible for the absence of oligomerization observed above. To address this question, we used HeLa cells, which express FLBax. To discriminate the exogenous from endogenous Bax, a GFP or a FLAG tag was fused to the amino terminus of FLBax or Bax/H9004C (see the “Experimental Procedures”). As shown in Fig. 4A, the expression of Bax in HeLa cells was similar in GFP-Bax and GFP-Bax/H9004C cells. However, we were able to obtain only a weak expression of the FLAG-Bax/H9004C construct (Fig. 4A). Nonetheless, the expression of Bax/H9004C constructs induced a rapid response to apoptosis when compared
with their wild-type counterparts (Fig. 4B). We could not obtain viable cells expressing FLAG-FLBax (data not shown), probably because the addition of the FLAG tag to the amino terminus of FLBax provoked a change in conformation that enhanced its apoptogenic properties (8, 16). On the other hand, we were able to obtain a stable expression of Bax constructs in which GFP was fused to the amino terminus of FLBax and BaxΔC. The subcellular localization of the resulting constructs (i.e. GFP-FLBax and GFP-BaxΔC) was assessed by laser confocal microscopy in HeLa cells. As shown in Fig. 4C, both constructs were found to be mostly mitochondrial, a result different from previous reports (23) but similar to that obtained in the HL60 cell line (8) or the endogenous Bax. In parallel, we analyzed the cross-linked products of untagged FLBax and BaxΔC in HeLa cells in the cytosolic and mitochondrial fractions of untreated and apoptotic cells (see “Experimental Procedures”). As shown in Fig. 5A, an analysis of the cross-linked products of untagged Bax in apoptotic (i.e. UV-B-treated) or healthy (i.e. untreated) samples showed that both constructs were present as monomers in the cytosol of healthy and apoptotic cells. FLBax oligomers (dimers and possibly trimers) were observed during apoptosis in nontransfected or in

FIGURE 5. A, HeLa cells were transfected with BaxΔC and the efficiency of the transfection analyzed by immunoblots with anti-Bax and anti-actin antibodies (left panel). Analysis of the oligomerization of Bax in the cytosol and in the mitochondria (Mito) isolated from control and BaxΔC-transfected apoptotic cells was performed using different cross-linkers (DSS or EGS) and Me2SO (DMSO) as a control. Bax complexes were analyzed by 12% SDS-PAGE and immunoblotting. The blots were washed and reprobed with an antibody against the mitochondrial protein TOM22 to control that equal amounts of mitochondrial protein were loaded in all lanes (not shown). Immunoblots are representative of at least three independent experiments. B, oligomerization of FLAG-BaxΔC was determined in the cytosol and in the mitochondria of UV-treated and untreated Du145 transfected cells by cross-linking followed by immunoblotting using an anti-FLAG antibody. Note that FLAG-BaxΔC formed no complexes in mitochondria after an UV treatment. C, BN-PAGE and immunoblotting analyses of Bax complexes present in mitochondria isolated from HeLa cells transfected with empty plasmid (mock) or FLBax in untreated (con) and UV-treated (apop) cells. For FLAG-BaxΔC, an anti-FLAG antibody was used. Immunoblots are representative of at least three independent experiments.

BN-PAGE allows the analysis of protein complexes by separating complexes essentially according to their size (24). BN-PAGE was used to examine the oligomerization status of FLBax and BaxΔC in mitochondria isolated from HeLa cells. As shown in Fig. 5C, Bax was present mainly as a low molecular weight complex (i.e. 21 kDa) in both fractions in healthy cells, and the molecular weight of the complexes increased during apoptosis. However, in both healthy and apoptotic cells, FLAG-BaxΔC, detected with an anti-FLAG antibody, presented a molecular organization consistent with a monomeric structure, although two bands were observed (Fig. 5C). Taken together, our results suggest that BaxΔC resides in mitochondria as monomers during apoptosis despite the presence of endogenous Bax in HeLa cells.
Bid- or Temperature-induced Oligomerization of Bax Is Abolished in Bax\textsuperscript{H9004}C Construct—We assayed the influence of the oligomerization of Bax in an acellular assay as described earlier (11). Briefly, in vitro translated (IVT)-Bax constructs labeled with \[^{35}\text{S}]\text{methionine} were activated by either raising the temperature or co-incubation with 8 fmol of rt-Bid. After the removal of rt-Bid, the presence of monomers or dimers of the various IVT-Bax constructs was determined under native or denaturing PAGE conditions. The incubation of FLBax or FLAG-FLBax with rt-Bid or at 43 °C induced the formation of dimers, and under similar conditions, Bax\textsuperscript{H9004}C or FLAG-Bax\textsuperscript{H9004}C remained monomeric (Fig. 6A). As expected, similar results were obtained with a Bax variant with a mutation in the BH3 domain (i.e., L63E), which has been shown to prevent oligomerization (25) (Fig. 6A). Interestingly, the BaxTM\textsuperscript{xL}, a construct in which the Bax-CT was replaced by its equivalent Bcl-xL segment, underwent a dimerization process when subjected to a change in temperature (43 °C) or the addition of rt-Bid. It should be added that BaxTM\textsuperscript{xL} has been shown to associate with mitochondria in vitro but was incapable of inducing apoptosis after transfection into HL60 cells or in yeast (26). These results suggest that the oligomerization of Bax can be triggered by another hydrophobic carboxyl terminus and that this oligomerization is not a prerequisite for its apoptotic function.

We also co-incubated IVT-Bax and unlabeled FLAG-Bax at 30 and 43 °C or with rt-Bid, and then the mixture was immunoprecipitated with anti-FLAG antibodies and analyzed under nondenaturating conditions. As shown in Fig. 6B, IVT-FLAG-FLBax formed dimers under these conditions. Similar results were obtained after incubation of IVT-FLAG-FLBax and FLBax. Inversely, after co-incubation of IVT-FLAG-Bax\textsuperscript{H9004}C and Bax\textsuperscript{H9004}C (or vice versa), immunoprecipitation with anti-FLAG antibodies demonstrated that Bax\textsuperscript{H9004}C could not self-associate under the same conditions (Fig. 6B).

The Bax-CT Is Not a Dimerization Domain—Our results show that a deletion of the Bax-CT interfered with Bax dimerization in vitro and in an acellular assay. We analyzed the cross-linked products formed upon the incubation of FLBax, Bax\textsuperscript{AC}, and RFP-H\textsubscript{9251}-9 constructs at pH 7.8, which is known to induce dimerization (10). As shown in Fig. 7A, the incubation of IVT-FLAG-FLBax at pH 7.8 induced its dimerization as assessed by cross-linking of the IVT construct, although it remained monomeric at pH 7.4. Conversely, under the same conditions, no dimers were observed with RFP-H\textsubscript{9251}-9 or Bax\textsuperscript{AC} (Fig. 7A). In addition, cytosolic Bax\textsuperscript{AC} obtained from Du145-transfected cells was treated with Triton X-100 under conditions that promote the
Indeed, the expression of Bax in mitochondria (Fig. 1), even though it is present as monomers in the cytosol (5). The ability of FLAG-BaxΔC to oligomerize with endogenous FLBax was assessed in HeLa cells (Fig. 4). BaxΔC translocation to mitochondria was observed using a GFP-BaxΔC construct. Indeed, contrary to previous reports, our GFP-BaxΔC construct translocated to mitochondria after the induction of apoptosis. The oligomerization of BaxΔC and FLAG-BaxΔC in HeLa cells was first analyzed by cross-linking cytosolic and mitochondrial proteins in both healthy and apoptotic cells and second by evaluating the molecular complexes formed by Bax in BN-PAGE and SDS-PAGE followed by immunodetection (Fig. 5). The data obtained showed that BaxΔC could not form oligomers with itself or with endogenous FLBax. We also used a cell-free assay in which the different Bax constructs, activated either with rt-Bid or by a rise in temperature, were incubated and the resulting complexes analyzed in BN-PAGE (Fig. 6). FLBax was capable of forming dimers under these conditions, whereas BaxΔC was present only as monomers. A similar result was obtained with the FLBax-L63E construct, which has been shown to be unable to homodimerize in vitro. Interestingly, the substitution of Bax Hα-9 by Bcl-xL Hα-8, which is involved in the association of Bcl-xL with mitochondria during apoptosis, restored the capacity of BaxΔC to homodimerize (Fig. 6). Interestingly, the co-incubation of BaxΔC with FLBax did not lead to the formation of heterodimers, suggesting that the presence of a carboxyl terminus in both partners was required (Fig. 6). One possibility implied by the latter results was that dimerization occurred directly through the carboxyl terminus as described for several other membrane proteins. This hypothesis was analyzed in the double hybrid assay in bacteria or by cross-linking and then determining the interaction between different Hα constructs. As shown in Fig. 7, the presence of Bax Hα-9 did not induce the oligomerization of soluble RFP, ruling out the involvement of a direct interaction of this Hα in Bax oligomerization. Exposure of FLBax to detergent induces the irreversible formation of high order oligomers. Of note, detergent-treated BaxΔC formed dimers demonstrating that it is not a prerequisite in the formation of dimers (Fig. 7).

Dimerization of Bax (27). Fig. 7B shows that detergent-treated BaxΔC was still capable of forming dimers suggesting that the Hα-9 only indirectly controls the dimerization process of Bax and that it is neither sufficient nor necessary for this process. This result suggests that it is possible that a small amount of BaxΔC oligomerizes to kill cells. One possible explanation of our results is that the carboxyl terminus of Bax is involved in the stabilization of Bax oligomers (Fig. 7C).

**DISCUSSION**

Inactive cytosolic Bax appears to be a globular monomeric protein in which the carboxyl terminus is closely associated with a hydrophobic pocket formed in part by the putative pore-forming Hα-5 and Hα-6 (28). Once activated at the onset of apoptosis, Bax undergoes major conformational changes, which promotes the addressing of Bax to the mitochondria and its insertion into the outer membrane of the organelle (4). Bax membrane integration is followed or closely associated with its oligomerization and AMOMP. The involvement of the carboxyl terminus in these processes has been controversial, but even if this segment is dispensable for the addressing and the integration of Bax into mitochondria, Hα-9 appears to be membrane-anchored in Bax-activated conformers (6). Of note, the latter step appears to be necessary for AMOMP and to require c-Myc expression (5).

The data presented here shows that the Bax-CT was involved in the oligomerization step but that monomers formed by BaxΔC are still capable of inducing AMOMP and cell death. Indeed, the expression of BaxΔC in DU145 is capable of inducing apoptosis and promoting cytochrome c release from mitochondria (Fig. 1), even though it is present as monomers in the mitochondria isolated from dying cells, whereas FLBax formed mainly dimers (Fig. 3). This result suggests that Bax-CT could be important for the formation of oligomers. The ability of FLBaxΔC to oligomerize with endogenous FLBax was assessed in HeLa cells (Fig. 4). BaxΔC translocation to mitochondria was observed using a GFP-BaxΔC construct. Indeed, contrary to previous reports, our GFP-BaxΔC construct translocated to mitochondria after the induction of apoptosis. The oligomerization of BaxΔC and FLAG-BaxΔC in HeLa cells was first analyzed by cross-linking cytosolic and mitochondrial proteins in both healthy and apoptotic cells and second by evaluating the molecular complexes formed by Bax in BN-PAGE and SDS-PAGE followed by immunodetection (Fig. 5). The data obtained showed that BaxΔC could not form oligomers with itself or with endogenous FLBax.
most biophysical studies suggest that only oligomers can exhibit pore forming activity. However, it should be noted that it has been shown that Bax homodimerization was not required to enhance cell death (25, 29) Perhaps it is possible that BaxΔC oligomers are not stable and thus cannot be observed under our experimental conditions. One can postulate that Bax-CT could be involved in dimerization of Bax in a reversible phase by facilitating the association of oligomers from smaller structures (or alternatively by inhibiting the dissociation of high molecular weight structure) (Fig. 7C). However, the relationship between Bax and Bak (Fig. 7C) and its importance in the homodimerization process remains to be investigated.

Of note, it has been shown that the carboxyl terminus of the anti-apoptotic protein Bcl-xL is involved in its oligomerization through an interaction with the BH3 domain (30, 31). Interestingly, Bcl-xL constructs, which lack the last 22 amino acids in the carboxyl terminus are predominantly present as monomers but are still capable of forming dimers at basic pH through a three-dimensional domain swapping (32). We have previously reported that Bax Hα-1 interferes with its BH3 domain (33), but it is possible that the formation of Bax dimers though the BH3 domain is under the tight control of its carboxyl terminus as described for Bcl-xL (31). Our results also provide an explanation for the divergent data published on the role of the carboxyl terminus as it is possible that in some cell lines or under certain apoptotic conditions the formation of oligomers is required for Bax membrane integration and/or induction of AMOMP, and thus BaxΔC would be inactive under these circumstances.

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