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Mutagenesis of the BH3 Domain of BAX Identifies Residues Critical for Dimerization and Killing

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The BCL-2 family of proteins is comprised of proapoptotic as well as antiapoptotic members (S. N. Farrow and R. Brown, Curr. Opin. Genet. Dev. 6:45–49, 1996). A prominent death agonist, BAX, forms homodimers and heterodimerizes with multiple antiapoptotic members. Death agonists have an amphipathic α helix, called BH3; however, the initial assessment of BH3 in BAX has yielded conflicting results. Our BAX deletion constructs and minimal domain constructs indicated that the BH3 domain was required for BAX homodimerization and heterodimerization with BCL-2, BCL-X_L, and MCL-1. An extensive site-directed mutagenesis of BH3 revealed that substitutions along the hydrophobic face of BH3, especially charged substitutions, had the greatest affects on dimerization patterns and death agonist activity. Particularly instructive was the BAX mutant mIII-1 (L63A, G67A, L70A, and M74A), which replaced the hydrophobic face of BH3 with alanines, preserving its amphipathic nature. BAXmIII-1 failed to form heterodimers or homodimers by yeast two-hybrid or immunoprecipitation analysis yet retained proapoptotic activity. This suggests that BAX's killing function reflects mechanisms beyond its binding to BCL-2 or BCL-X_L to inhibit them or simply displace other protein partners. Notably, BAXmIII-1 was found predominantly in mitochondrial membranes, where it was homodimerized as assessed by homobifunctional cross-linkers. This characteristic of BAXmIII-1 correlates with its capacity to induce mitochondrial dysfunction, caspase activation, and apoptosis. These data are consistent with a model in which BAX death agonist activity may require an intramembranous conformation of this molecule that is not assessed accurately by classic binding assays.

Programmed cell death and its morphologic equivalent, apoptosis, are orchestrated by a distinct genetic pathway that is apparently possessed by all multicellular organisms (22). Moreover, the biochemical details of how encoded proteins function are beginning to emerge. The BCL-2 family of proteins constitutes a central decisional point within the common portion of the apoptotic pathway. This family possesses both proapoptotic (BAX, BAK, BCL-X_S, BAD, BIK, BID, HRK, and BIM) and antiapoptotic (BCL-2, BCL-X_L, MCL-1, and A1) molecules (5, 11). The ratio of antiapoptotic to proapoptotic molecules such as BCL-2/BAX determines the response to a proximal apoptotic signal (14). A striking characteristic of many family members is their propensity to form homo- and heterodimers (16, 19). The BCL-2 family has homology clustered principally within four conserved domains called BH1, BH2, BH3, and BH4 (5, 11). The multidimensional nuclear magnetic resonance (NMR) and X-ray crystallographic structure of a BCL-X_L monomer indicates that the BH1-4 domains correspond to α helices 1 to 7. Notably, the BH1, -2, and -3 domains are in close proximity and create a hydrophobic pocket presumably involved in interactions with other BCL-2 family members (13). The NMR analysis of a BCL-X_L-BAK BH3 peptide complex revealed both hydrophobic and electrostatic interactions between the BCL-X_L pocket and a BH3 amphipathic α-helical peptide from BAK (17).

Prior mutagenesis studies of BCL-2 and BCL-X_L revealed the importance of BH1 and BH2 domains for both their antiapoptotic function and the capacity to heterodimerize with proapoptotic molecules like BAX or BAK (2, 19, 26). In general, most mutations that disrupt heterodimerization with BAX also lose their death repressor function. However, exceptions do exist; some mutants of BCL-X_L fail to bind BAX or BAK but still repress cell death, suggesting that these functions can be separated for antiapoptotic molecules (2). Moreover, a genetic approach with *Bcl-2*-deficient and *Bax*-deficient mice also suggested that BCL-2 and BAX could function independently of one another (10).

Deletion studies of the death agonist BAK first implicated the BH3 domain as having the capacity to bind BCL-X_L and promote apoptosis (3). However, the functional significance of BH3 in BAX is uncertain as indicated in the literature. Three deletion analyses indicated the necessity of the BH3 domain in BAX to promote cell death as well as to heterodimerize with BCL-2 (3, 9, 28). Yet, two recent studies reported that BAX functions as a death activator independent of its heterodimerization (21, 27). Moreover, substitution mutants within the BH3 domain showed conflicting specificities of heterodimerization (20, 21, 27).

Our initial screen of yeast two-hybrid libraries with BCL-2 as bait yielded multiple clones that possess only the NH_2 terminus of BAX, bearing the BH3 but not the BH1 or the BH2 domains. A similar set of isolates was obtained when BCL-2 (G145A) was used as bait (15). We also noted by deletion analysis and assessment of minimal domains of BAX that the BH3 domain was required for both homodimerization and heterodimerization. Consequently, we undertook an extensive site-directed mutagenesis of the BH3 domain of BAX. These studies demonstrate the importance of the hydrophobic face of the amphipathic α helix of BH3 for the dimerization and cell death activities of BAX. Furthermore, analysis of a BAX mutant indicates that its retained conformation as a cross-linkable

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dimer at mitochondrial membranes correlates with its intact apoptotic function.

MATERIALS AND METHODS

Construction of *Bax* BH3 domain mutants. Constructs expressing mutant BAX bearing a hemagglutinin (HA) tag at the NH_2 terminus or lacking the COOH-terminal hydrophobic segment (ΔC) were generated by two-step PCR with a unique *Pst*I site at 280 bp of the murine *Bax* open reading frame. A pSFFV-HABax plasmid (14) was used as the template. First, the 5′ 280 bp of *Bax* cDNA plus the HA tag were PCR amplified by a 5′ primer possessing an *Eco*RI site and a 3′ primer containing a *Pst*I site and the introduced mutation. This amplified *Eco*RI/*Pst*I fragment plus a *Pst*I/*Eco*RI fragment that completed the coding region were ligated into the *Eco*RI site of the DNA-binding domain (DBD) vector pBTM116 (19). The constructs were sequenced to confirm the presence of introduced mutations. Subsequently, the entire insert was subcloned into pSFFV or pcDNA3 (Invitrogen). A new pair of primers (5′-TTAGAATT CTAATGGACGGGTCCGGGGAG, CCCACATGGCAGACAGTGTGACTC GAGTTA-3′) was used to PCR amplify the *Bax* mutants, and the *Eco*RI/*Xho*I fragment was subcloned in frame into the activation domain (AD) vector pACTII and bacterial expression vector pGEX-4T-2 (Pharmacia Biotech Inc.) between the *Eco*RI and *Xho*I sites.

Yeast two-hybrid assay. pBTM-HABaxΔC wild type (wt) and mutants were cotransformed with Bcl-x_LΔC, Bcl-2ΔC, BaxΔC, or Mcl-1ΔC in pACTII into yeast strain L40. Transformants growing on plates without Trp or Leu were transferred onto NitroPure filters (Micron Separations Inc.) and assayed for β-galactosidase (β-Gal) activity as described previously (19).

Immunoprecipitation (IP) and Western blot hybridization. Cells were lysed in 100 μl of Nonidet P-40 (NP-40) isotonic lysis buffer with freshly added protease inhibitors (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.2], 1 mM EDTA, 0.25% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml), incubated on ice for 30 min, and centrifuged at 15,000 × *g* for 10 min to precipitate nuclei and nonlysed cells. Ten micrograms of anti-HA or anti-BCL-2 monoclonal antibodies (MAbs) was added to the supernatant of each sample, mixed, and incubated on ice for 30 min. Then 400 μl of NP-40 buffer was added along with 25 μl of protein A-Sepharose beads and incubated at 4°C with nutation for 1 to 2 h. Immunoprecipitates were collected by a brief spin, washed three times with 1 ml of NP-40 buffer, and solubilized with 1× sample buffer. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine gels (NOVEX) and transferred to polyvinylidene difluoride membranes (BioTrace; Gelman Sciences). Filters were blocked overnight at 4°C with Tris-buffered saline plus 0.1% Tween 20 (TBST) containing 6% nonfat milk. Subsequently, filters were incubated with primary and secondary Abs for 1 h each and washed in TBST for 5 min × 3, and developed by enhanced chemiluminescence (Amersham).

Transient transfection in Rat-1 and 293T cells. Experiments were performed as previously described (23). Briefly, Rat-1 cells were grown to about 80% confluence in 12-well plates before transfection. The luciferase reporter plasmid (0.1 μg) was mixed with 0.05 μg of various constructs as indicated and 3 μl of LipofectAMINE (Gibco BRL) in a volume of 0.5 ml per transfection. Lipofection was carried out as suggested by the manufacturer (Gibco BRL) for 5 h. Cells were lysed 18 to 20 h later, and a luciferase assay was performed with luciferase substrates from Promega. Luciferase activity was determined with a luminometer (Optocomp II; MGM Instruments Inc.). Cell viability is presented as relative luciferase activity compared to a control transfection. Transfection of 293T cells was carried out as described above except that cell lysates were used for IP and Western blot hybridization.

Mitochondrial membrane potential ($\Delta\Psi_m$) and reactive oxygen species (ROS) measurement. Experiments were performed as previously described (25). Briefly, 5×10^5 cells were incubated for 15 min at 37°C with 40 nM 3,3′-dihexyloxacarbocynine iodide ($DiOC_6$; Molecular Probes) or 2 μM hydroethidine (Molecular Probes), followed by flow cytometry with a fluorescence-activated cell sorter (Becton Dickinson).

Caspase activity assay. Cells were lysed in buffer containing 25 mM HEPES (pH 7.5), 5 mM EDTA, 2 mM dithiothreitol, and 10 μM digitonin. The lysates were clarified by centrifugation, and enzymatic reactions were carried out with 20 μg of protein and 50 μM acetyl-Asp-Glu-Val-Asp-aminotrifluoromethylcovmarin (DEVD-AFC; Enzyme System Products, Livermore, Calif.). The reaction mixtures were incubated at 37°C for 30 min, and the fluorescent AFC formation was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm with an FL500 microplate fluorescence reader (Bio-Tek, Burlington, Vt.).

Subcellular fractionation. Jurkat cells (4×10^7) were collected by centrifugation, washed in phosphate-buffered saline, and resuspended in isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES [pH 7.5]) supplemented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml). Cells were homogenized with a polytron homogenizer (Brinkmann Instruments) at setting 6.5 for 10 s. Nuclei and unbroken cells were collected by centrifugation at 120 × *g* for 5 min as the low-speed pellet (P1). The supernatant was centrifuged at 10,000 × *g* for 10 min to collect the heavy membrane (HM) fraction. Light membrane (LM) fraction was collected by centrifugation at 100,000 × *g* for 30 min with a TL-100 ultracentrifuge (Beckman Instruments). The supernatant, which contains mostly cytosolic proteins, was labeled as the soluble fraction (S100).

Cytochrome *c* release. Subcellular fractions of Jurkat cells were separated by SDS-PAGE on 16% Tris-glycine gels and transferred to polyvinylidene difluoride membranes. Blots were hybridized with anti-cytochrome *c* MAb 65981A (PharMingen) and developed by enhanced chemiluminescence.

Cross-linking. The HM fraction (0.5 mg of protein) was resuspended in the isotonic buffer and Bis (sulfosuccinimidyl) suberate (BS^3) (Pierce) in 5 mM sodium citrate buffer, pH 5.0, or disuccinimisyl suberate (DSS) (Pierce) in dimethyl sulfoxide was added to a final concentration of 10 mM. After incubation for 30 min at room temperature, the cross-linker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM. Subsequently, membranes were lysed in radioimmunoprecipitation assay buffer and cleared by centrifugation at 12,000 × *g*. Lysates were separated on SDS–12% PAGE gels followed by Western blot hybridization.

RESULTS

BH3 domain of BAX is critical for dimerization. To identify additional interacting proteins, we utilized BAX and BCL-2 as bait in a yeast two-hybrid-based screen of a mouse embryonic day 9.5 library. Multiple clones encoding truncated forms of BAX were isolated from these screens. These truncated clones revealed that the first 104 amino acids (aa) of BAX, which do not contain either the BH1 or the BH2 domain, were sufficient for dimerization with BAX or BCL-2 (data not shown). To further characterize the minimal domain of BAX required for dimerization, a series of truncations were generated and fused to LexA in the DNA-binding domain (DBD) vector pBTM. These BAX truncations were tested against BAX, BCL-2, BCL-X_L, and MCL-1 (without their COOH-terminal hydrophobic segments) in the activation domain (AD) vector pACTII. The most NH_2-terminal portion of BAX (aa 1 to 63) failed to interact with BAX, BCL-2, BCL-X_L or MCL-1, while the portion with 14 additional amino acids, BAX1–77, interacted with all four proteins (Fig. 1). This indicated that the BAX fragment aa 64 to 77 possessing the BH3 domain was necessary for dimerization with multiple partners. A BAX construct consisting of only aa 54 to 77 also proved capable of interacting with all four family members tested. These results implicate the BH3 domain as the minimal region required for dimerization.

Mutagenesis of BAX BH3 domain. To further investigate the role of the BH3 domain for BAX function and dimerization, we performed a systematic amino acid substitution analysis of this amphipathic α helix. The 15 BH3 mutants are defined in Fig. 2A. These BAX mutants were initially analyzed by yeast two-hybrid analysis, the results of which are summarized in Table 1. All mutants except BAXmIII-1 (L63A, G67A, L70A, and M74A) and BAXmIII-2 (L63E) retain the ability to interact with wild-type (wt) BAX. This identifies these four residues on the hydrophobic face of the α helix (Fig. 2B) as critical for the formation of BAX homodimers in this system. Single alanine replacement within the core of the conserved BH3 motif including BAXmIII-9 (D68A), BAXmIII-10 (E69A), BAXmIII-11 (L70A), and BAXmIII-12 (D71A) mutants (Fig. 2) displayed reduced interaction with BCL-X_L (Table 1). BAXmIII-4 (G67E) and BAXmIII-5 (M74A) lost interaction with both BCL-2 and BCL-X_L. In contrast, BAXmIII-3 (G67A), BAXmIII-6 (L63A), BAXmIII-7 (R64A and R65A), BAXmIII-8 (I66E), BAXmIII-13 (S72A), BAXmIII-14 (N73A), and BAXmIII-15 (E75A) displayed no alteration in their dimerization capacity in this assay (Table 1).

To further assess the accuracy of the yeast two-hybrid data, we analyzed the protein interactions of five instructive BAX BH3 mutants in mammalian cells. HA-tagged versions of BH3 mutants mIII-1 to -5 and wt *Bcl-2* were transiently transfected

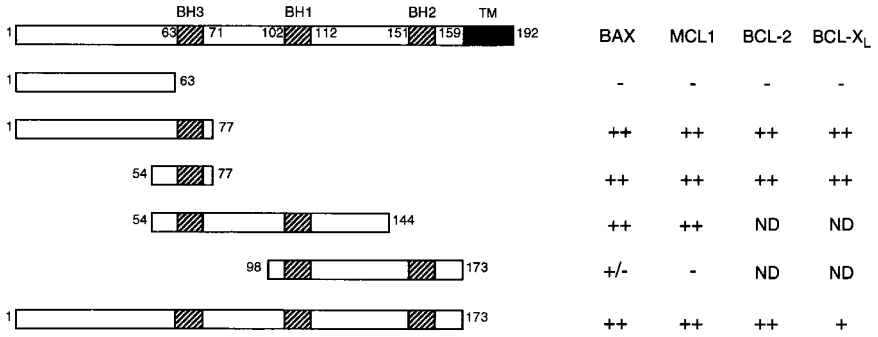

FIG. 1. Deletion analysis of BAX by yeast two-hybrid system. Various BAX NH_2- or COOH-terminal truncations in DBD vector pBTM were tested against BAX, BCL-2, BCL-X_L, and MCL-1 in the AD vector pACTII. Pairs of DBD and AD vectors were transformed into yeast strain L40 and plated on medium without Trp or Leu. Grown-up colonies were transferred onto NitroPure filters and assayed for β-Gal activity. −, no interaction; ±, weak interaction; + and ++, strong interaction; ND, no data.

into 293T cells. Coimmunoprecipitation of cell lysates with anti-HA or anti-BCL-2 Abs corroborated most of the interactions noted in the yeast two-hybrid system (Fig. 3A). One prominent exception was BAXmIII-5 (M74A), which coimmunoprecipitated with BCL-2 and thus formed heterodimers with BCL-2 as well as homodimers in mammalian cells. Overall, these mutants were classifiable into three generalized groups: BAXmIII-1 and -2, which bind neither BAX nor BCL-2; BAXmIII-4, which binds BAX but not BCL-2; and BAXmIII-3 and -5, which bind BAX and BCL-2.

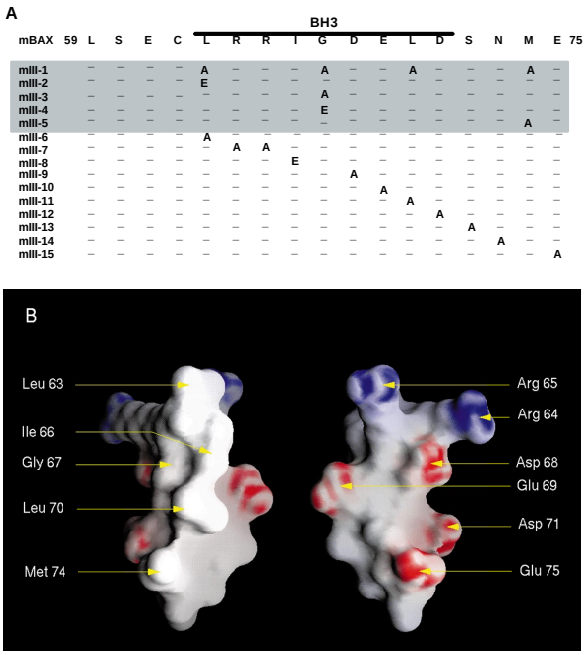

FIG. 2. Mutagenesis and three-dimensional modeling of the BH3 domain of BAX. (A) Schematic representation of mutations in the BH3 domain of BAX. (B) Model of the molecular surface of BAX BH3, calculated and displayed by GRASP (13). The surface is colored deep blue (23 kBT) in the most-positive regions and deep red (−21 kBT) in the most negative, with linear interpolation for values in between. This model was generated with the protein building module (BUILDER) of INDIGHTII (Biosym, San Diego, Calif.) and minimized with DISCOVER, the force-field simulation module of INSIGHTII. Left, view of the hydrophobic surface of the amphipathic BH3 helix of BAX. Right, view of the polar surface of the amphipathic BH3 α-2 helix of BAX. Residues forming polar and hydrophobic surfaces are indicated.

Functional analysis of BAX mutants. To investigate the death-inducing activity of the BAX BH3 mutants, we used a transient transfection system in Rat-1 fibroblasts as described previously (23). *Bax* mutants in a mammalian expression vector were cotransfected with a luciferase reporter into Rat-1 cells. Luciferase activity was assessed 16 to 18 h after transfection, and its decrease has been shown to parallel the loss of viability (23). Cotransfection of wt *Bax* with the luciferase reporter resulted in a 10-fold decrease in luciferase activity (Fig. 4A). BAX mutants mIII-1, -3, and -5 retained nearly wt killing activity, while mutants 2 and 4 were six- and threefold-less-potent than wt BAX, respectively (Fig. 4A).

To assess the ability of BAX BH3 mutants to counteract the antiapoptotic effect of BCL-2, we cotransfected the mutants together with *Bcl-2*. Transfection of wt *Bcl-2* results in an increase in luciferase activity of approximately threefold, reflecting BCL-2's protection from lipofection-induced cell death (Fig. 4B). Cotransfection of wt *Bax* with *Bcl-2* decreased the luciferase activity, confirming the capacity of BAX to counteract BCL-2. BAXmIII-1 and -5 retained full capacity to counter

TABLE 1. Yeast two-hybrid analysis of the protein interactions of BAX BH3 mutants[a]

BAX wt or mutant construct in DBD vector pBTM	Protein interaction with indicated construct in AD vector pACTII[b]		
	BAX	BCL-2	BCL-X_L
BAXwt	+	+	+
mIII-1	−	−	−
mIII-2	−	−	−
mIII-3	+	+	+
mIII-4	±	−	−
mIII-5	+	−	−
mIII-6	+	+	+
mIII-7	+	ND	+
mIII-8	+	+	+
mIII-9	+	+	±
mIII-10	+	+	±
mIII-11	+	+	−
mIII-12	+	+	−
mIII-13	+	+	+
mIII-14	+	+	+
mIII-15	+	+	+

[a] All constructs lack COOH-terminal signal anchor (ΔC). Results reflect β-Gal assays from at least two independent experiments.

[b] +, strong interaction; −, no interaction; ±, weak interaction; ND, no data.

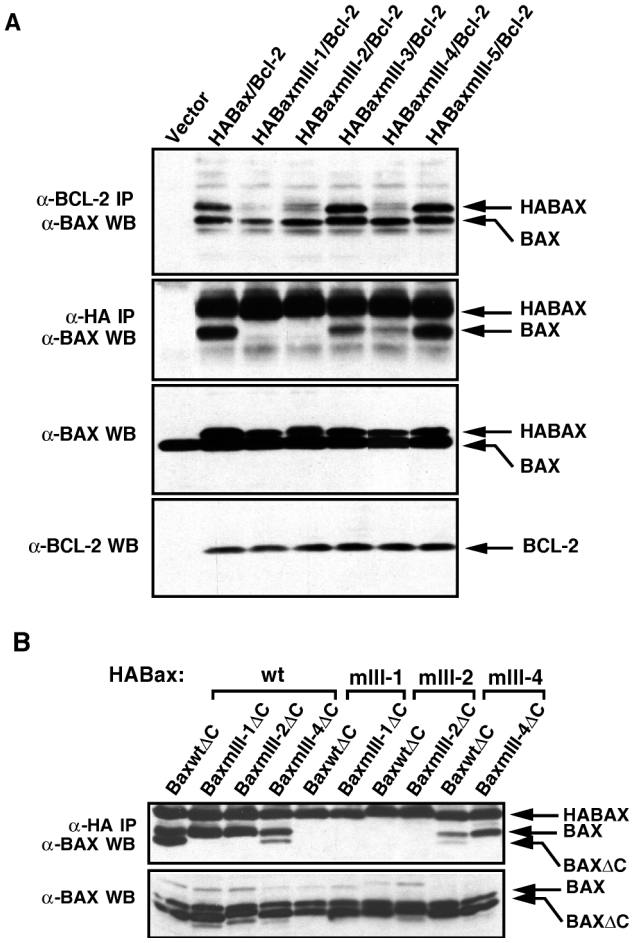

FIG. 3. Interaction of BAX BH3 mutants in a transient transfection system. (A) NP-40-solubilized lysates from 293T cells transfected with *Bax* mutants and *Bcl-2* were immunoprecipitated with 6C8 anti-BCL-2 or 12CA5 anti-HA MAbs. Immunoprecipitates (first panel, anti-BCL-2 IP; second panel, anti-HA IP) or direct cell lysates (third and fourth panels) were size fractionated onto 16% Tris-glycine SDS-PAGE gels, followed by the development of Western blots with the N20 anti-BAX Ab (Santa Cruz Biotechnology) or 6C8 anti-BCL-2 MAb. (B) NP-40-solubilized lysates from 293T cells cotransfected with Bax mutants that were either tagged with HA or lacking the COOH-terminal hydrophobic segment were immunoprecipitated with 12CA5 anti-HA MAb, followed by the same steps as those described for panel A. Upper panel, results of the IP-Western analysis; lower panel, results of Western analysis of total cell lysates.

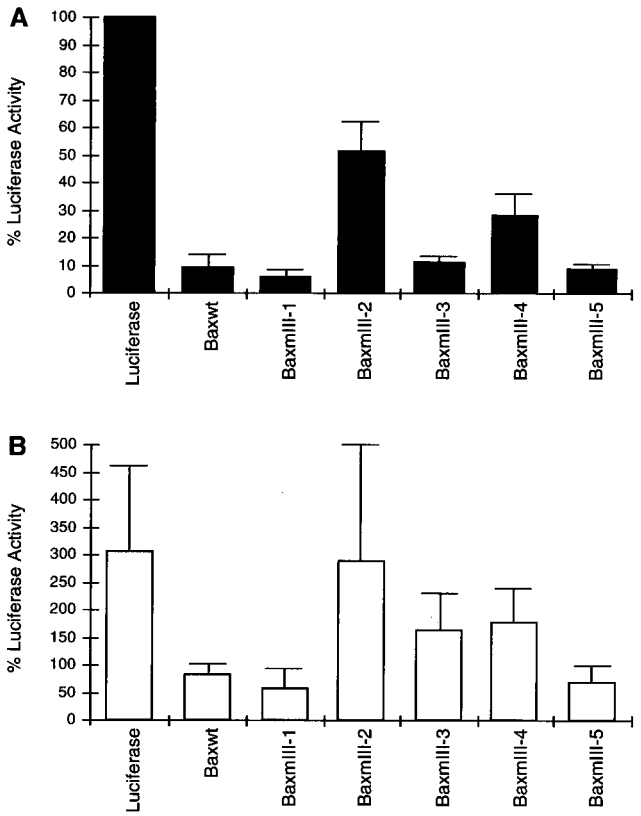

FIG. 4. BAX mutant-induced apoptosis in Rat-1 cells. Rat-1 cells were cotransfected with a luciferase reporter and either *Bax* (A) or both *Bax* and *Bcl-2* (B) in pcDNA3, mediated by LipofectAMINE. Cell lysates were collected 16 to 18 h after transfection and assayed for luciferase activity. Luciferase activity for each assay is presented as the percentage of a control transfection in which a reporter plus an empty pcDNA3 vector were transfected. Results shown are means ± standard deviations (error bars) from three independent experiments.

BCL-2. BAXmIII-3 and -4 were partially impaired in countering BCL-2. BAXmIII-2, which was the most disabled in its singular agonist activity, also lost the ability to reverse BCL-2 protection (Fig. 4B and Table 2).

Comparison of apoptotic events induced by the expression of wt BAX versus BAXmIII-1. The BAXmIII-1 mutant, which replaced the hydrophobic face of the BH3 α helix (Fig. 2B) with alanines, induced apoptosis and counteracted BCL-2 with an efficiency comparable to that of wt BAX in transient death assays. This provides an instructive example, as mIII-1 had lost the capacity to dimerize with wt BAX and BCL-2 by yeast two-hybrid and IP assays. The doxycycline-induced expression of wt BAX in the Jurkat T-cell line resulted in altered mitochondrial membrane potentisl ($\Delta\Psi_m$), production of ROS, activation of caspases, and apoptotic cell death (25). In order to investigate the downstream death program of BAXmIII-1, we generated a comparable Jurkat clone bearing an HA-tagged BAXmIII-1 molecule under the control of doxycycline. Following induction, BAXmIII-1 killed cells to the same extent in a time course comparable to that of wt BAX (Fig. 5A), confirming the impression by the transient death assays in Rat-1 cells (Fig. 4). Furthermore, the time course of the loss of $\Delta\Psi_m$ as determined by $DiOC_6$ (Fig. 5B), the production of ROS as assessed by hydroethidine (Fig. 5C), and the activation of caspases as assessed by the cleavage of a tetrapeptide-fluorochrome substrate (DEVD-AFC; Fig. 5D) were very similar between BAX wt and mIII-1 clones. Despite this evidence of mitochondrial dysfunction and caspase activation, neither wt BAX nor BAXmIII-1 released a substantial amount of cytochrome *c* in a 48-h period during which the onset of these aberrations occurred (Fig. 5E).

Assessment of the capacity of BAX mutants to form mutant-mutant homodimers by yeast two-hybrid and immunoprecipitation. The intact death effector activity of mutant protein BAXmIII-1 provided an apparent dissociation between dimerization capability and killing in that it failed to dimerize with either wt BAX or BCL-2. However, a remaining possibility was that the BAXmIII-1 molecule had the ability to bind to itself as a mutant-mutant homodimer. To assess this possibility, we cloned each mutant BAX (mIII-1 to -5) into both the AD vector and DBD vector and analyzed dimerization by a yeast two-hybrid test (Table 2). BAXmIII-1, -2, and -4 failed to demonstrate mutant-mutant self-dimerization.

The capacity of these mutants to form homodimers was also

TABLE 2. Summary of BAX mutants in the BH3 domain[a]

BAX wt or mutant construct	Yeast two-hybrid interaction with:			In vivo interaction with:			Death agonist activity	Capacity to counteract BCL-2
	BAX	BCL-2	BAXmut	BAX	BCL-2	BAXmut		
BAX wt	+	+	NA	+	+	NA	+	+
mIII-1	−	−	−	−	−	−	+	+
mIII-2	−	−	−	−	−	−	+	−
mIII-3	+	+	+	+	+	+	+	±
mIII-4	±	−	−	±	−	−	±	±
mIII-5	+	−	+	+	+	+	+	+

[a] −, inactive; ±, partially active; +, active; NA, not applicable.

examined by transient expression and coimmunoprecipitation in 293T cells. In this paradigm, constructs of BAX mutants (mIII-1, -2, and -4) lacking the COOH-terminal hydrophobic segment were coexpressed with their corresponding HA-tagged versions. Immunoprecipitation of the HA-tagged proteins from NP-40-solubilized lysates was followed by the development of Western blots with an anti-BAX Ab. Under these conditions mIII-1, -2, and -4 did not form mutant-mutant homodimers (Fig. 3B), consistent with the data from the yeast two-hybrid assay (Table 2). This co-IP approach also demonstrated the capacity of BAXmIII-4 (G67E) to form dimers with wt BAX, albeit with decreased efficiency (Fig. 3B and Table 2).

BAXmIII-1 localizes to mitochondrial membranes where it can be cross-linked as a BAX homodimer. Recently, BAX has been shown to translocate from cytosol to mitochondrial membranes during apoptosis induced by staurosporine (8, 24), dexamethasone, γ-irradiation (24), or interleukin-3 withdrawal (6). Homobifunctional protein cross-linkers revealed that the

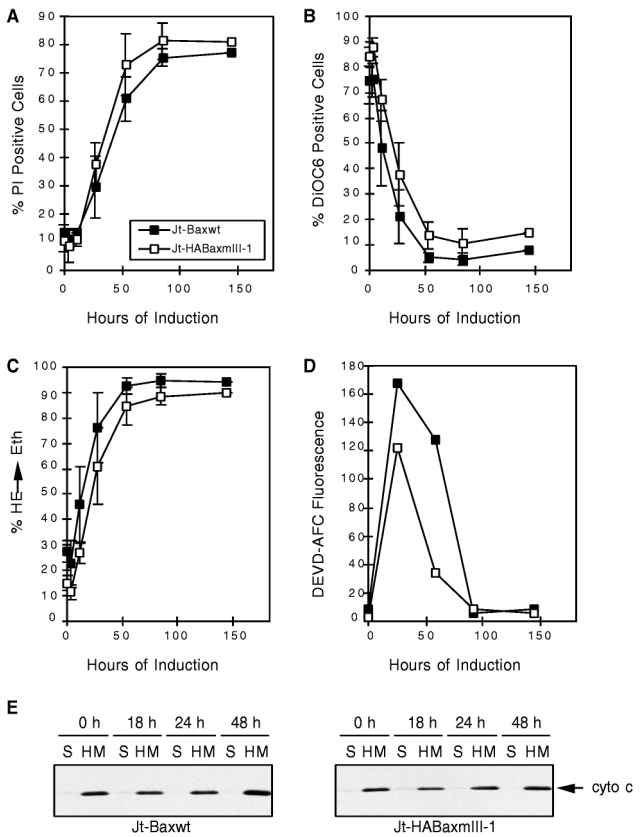

FIG. 5. BAX-induced apoptosis in Jurkat cells. Jurkat clones with inducible expression of wt BAX or HABAXmIII-1 were treated with 1 μg of doxycycline per ml and assayed for PI exclusion (A), mitochondrial-membrane potential (B), and ROS production (C) at the indicated time points. Data shown are means ± standard deviations (error bars) from three independent experiments. (D) Caspase activity was analyzed with fluorescent peptide substrate DEVD-AFC. (E) Release of cytochrome *c* (cyto c) was assessed by Western blot hybridization of subcellular fractions with an anti-cytochrome *c* MAb. HE, hydroethidine; Eth, ethidium.

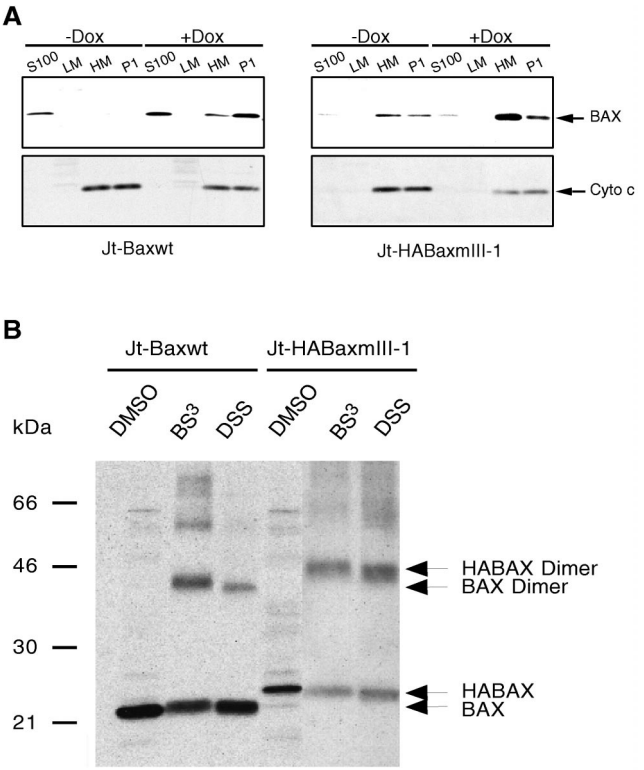

FIG. 6. Subcellular localization and homodimer formation of BAX in Jurkat cells. (A) Subcellular localization of BAX wt and mIII-1 in Jurkat cells. Jt-Baxwt and Jt-HABaxmIII-1 cells before and 24 h after doxycycline (Dox) treatment were suspended in isotonic buffer, homogenized with a polytron homogenizer, and separated into soluble fraction (S100), LM fraction, HM fraction, and low-speed pellet (P1) by differential centrifugation. The fractions were analyzed by Western blot hybridization with anti-BAX (N20), anti-HA (12CA5), or anti-cytochrome *c* (Cyto c) Abs. (B) HMs prepared from Jurkat cells after 24 h of treatment with 1 μg of doxycycline per ml were incubated in isotonic buffer and treated with membrane-impermeable BS^3 or membrane-permeable DSS cross-linkers or with dimethyl sulfoxide (DMSO) as a control. After treatment, membranes were lysed, cleared by centrifugation, and separated by SDS-PAGE, followed by Western blot hybridization with anti-BAX or anti-HA Abs. At the left are molecular size markers (in kilodaltons).

mitochondrial membrane-based BAX forms homodimers (6). Moreover, enforced homodimerization of BAX induced its translocation to mitochondria and resulted in apoptosis (6). The small amount of BAX wt present in Jurkat cells before induction was found in the cytosolic fraction (Fig. 6A). After induction, BAX appeared in the mitochondrion-enriched HM fraction and in the low-speed pellet (P1) comprised of residual whole cells, nuclei, and mitochondria (Fig. 6A). In contrast, BAXmIII-1, which was present principally in the HM and P1 fractions, displayed a marked increase in mitochondrial localization following induction (Fig. 6A). The mitochondrial BAXmIII-1, like wt BAX, proved resistant to alkaline extraction, indicating an integral membrane position (data not shown).

In order to assess the conformation of mitochondrial membrane-based BAXmIII-1, we utilized homobifunctional cross-linkers. Intact mitochondria were treated with the membrane-impermeable bis(sulfosuccinimidyl)suberate (BS^3) or membrane-permeable disuccinimidyl suberate (DSS) noncleavable primary amine cross-linker (Fig. 6B). A substantial portion of HABAXmIII-1 could be cross-linked as a homodimer with an apparent molecular size of 44 kDa, similar to the 42-kDa homodimer formed by wt BAX (Fig. 6B). This result contrasts with the inability of BAXmIII-1 to form homodimers by yeast two-hybrid and detergent-based co-IP assays (Fig. 3B and Table 2), suggesting that this intramembrane conformation is not reflected in those assays.

DISCUSSION

The importance of a minimal BH3 domain 54 to 77 aa from BAX in mediating both homo- and heterodimerization, together with the deletional analysis here and in prior studies (3, 7, 9, 20, 21, 27, 28), indicates that BH3 is the critical domain of BAX involved in both types of dimerization. This is also consistent with the capacity of BAX-derived BH3 peptides to block both homodimerization and heterodimerization of BCL-2 family members (4). BH3-deleted BAX molecules also showed impaired killing activity in previous studies (3, 9, 27) and in our own analysis (19a). However, no systematic mutagenesis of the BH3 domain of BAX had been performed to pinpoint the amino acids responsible for dimerization and determine how mutants of these amino acids would affect the death effector function.

The structure of the BCL-X_L monomer (13) prompted molecular modeling of the BAX BH3 domain, which revealed an amphipathic α helix. Our mutational analysis of BH3 indicates that substitution along the hydrophobic face with a charged residue, such as glutamic acid, alters the dimerization pattern, while substitution with a hydrophobic residue (alanine) may not. For example, mIII-2 (L63E) and -4 (G67E) both display altered dimerization, yet mIII-6 (L63A) and -3 (G67A) do not. BAXmIII-5 (M74A) did show loss of interaction with BCL-2 by a yeast two-hybrid assay, but the interaction was intact by IP in mammalian cells. BAXmIII-1, which has four alanine substitutions on the hydrophobic face of BH3, altered enough critical contacts along that binding surface to disrupt dimerization. In contrast, substitutions of the polar surface had a weaker effect on dimerization. For example, BAX mutants mIII-7, -8, -9, -10, -14, and -15 all displayed a normal pattern of homo- and heterodimerization. Only BAXmIII-12 (D71A) displayed the loss of BCL-X_L but not BCL-2 or BAX binding. This may indicate that the hydrophobic interactions at the base of the binding pocket are more important than the electrostatic interactions. In total, the yeast two-hybrid data also argue for a difference in the strength of the interactions between BAX and its partners. The BCL-X_L/BAX heterodimer was the most sensitive to mutations, while the BAX/BAX homodimer was least-often affected.

The analysis of a subset of the BAX BH3 mutants in death effector assays suggests that the maintenance of the amphipathic nature of the α-2 helix, BH3, is critical for BAX to kill. BAXmIII-1 with four alanine substitutions along the hydrophobic face of BH3, which would maintain the amphipathic nature of the α helix, displayed unimpaired death agonist activity. In contrast, mIII-2 (L63E) and mIII-4 (G67E) substituted a charged amino acid on this face, and both displayed diminished death effector function. Another naturally occurring missense mutation in the BH3 domain of BAX, G67R, was identified in a human leukemic cell line and classified as a loss-of-function mutation (12). Thus, the introduction of a charged residue on this hydrophobic face appears to be particularly deleterious to the killing function of BAX.

The BAXmIII-1 mutant proved most instructive because of its intact killing activity despite the loss of homo- and heterodimerization by yeast two-hybrid and detergent-based IP assays. This even raised the possibility that BAX monomers might be functionally active, which was also suggested by two independent studies (20, 27). However, a detailed analysis of the BAXmIII-1 mutant provides an alternative explanation for this apparent dilemma. BAXmIII-1 is localized predominantly to the mitochondrial membrane and in that setting could be cross-linked as a homodimer similar to wt BAX. A plausible explanation for this discrepancy is that BAX changes its conformation when it is inserted into membranes and that BAXmIII-1 has the capacity to assume this homodimerized conformation within membranes. The ability of BAXmIII-1 to localize to mitochondrial membranes and form homodimers may relate to its retained ability to induce mitochondrial dysfunction, caspase activation, and cell death. These findings with BAXmIII-1 lend support to the observation that enforced dimerization of BAX with an FKBP/FK1012 system was sufficient to induce mitochondrial dysfunction and cell death (6). In both systems, BAX-induced caspase activation and mitochondrial dysfunction occur without a demonstrable release of cytochrome *c*, suggesting that it may not be required for BAX-induced death. The retained capacity of the functionally active mutant BAXmIII-1 as well as wt BAX to form homodimers at the mitochondrial membrane may relate to the ability of BAX to form distinct ion-conductive channels (1, 18). The precise role of such pores in vivo is uncertain, but these data suggest that they may not directly release cytochrome *c*. The importance of the amphipathic nature of the α-2 helix, BH3, for the death effector function of BAX suggests that this helix is critical for initiating events at the mitochondrial membrane that lead to cell death.

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