Title
Heterodimerization-independent Functions of Cell Death Regulatory Proteins Bax and Bcl-2 in Yeast and Mammalian Cells

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The pro-apoptotic protein Bax can homodimerize with itself and heterodimerize with the anti-apoptotic protein Bcl-2, but the significance of these protein-protein interactions remains unclear. Alanine substitution mutations were created in a well conserved IGDE motif found within the BH3 domain of Bax (residues 66–69) and the resulting mutant Bax proteins were tested for ability to homodimerize with themselves and to heterodimerize with Bcl-2. Correlations were made with cell death induction by these mutants of Bax both in mammalian cells where Bax may function through several mechanisms, and in yeast where Bax may exert its lethal actions through a more limited repertoire of mechanisms perhaps related to its ability to form ion channels in intracellular membranes. Two of the mutants, Bax(D68A) and Bax(E69A), retained the ability to homodimerize but failed to interact with Bcl-2 as determined by yeast two-hybrid assays and co-immunoprecipitation analysis using transfected mammalian cells. The Bax(E69A) protein exhibited a lethal phenotype in yeast, which could be specifically suppressed by co-expression of Bcl-2, despite its failure to dimerize with Bcl-2. Both the Bax(D68A) and Bax(E69A) proteins induced apoptosis when overexpressed in human 293 cells, despite an inability to bind to Bcl-2. Moreover, co-expression of Bcl-2 with Bax(D68A) and Bax(E69A) rescued mammalian cells from apoptosis. In contrast, a mutant of Bax lacking the IGDE motif, Bax(AIGDE), was incapable of either homodimerizing with itself or heterodimerizing with Bcl-2 and was inactive at promoting cell death in either yeast or mammalian cells. Although failing to interact with Bcl-2, the Bax(D68A) and Bax(E69A) mutants retained the ability to bind to Bid, a putative Bax-activating member of the Bcl-2 family, and collaborated with Bid in inducing apoptosis. When taken together with previous observations, these findings indicate that (i) Bax can induce apoptosis in mammalian cells irrespective of heterodimerization with Bcl-2 and (ii) Bcl-2 can rescue both mammalian cells and yeast from the lethal effects of Bax without heterodimerizing with it. However, these results do not exclude the possibility that BH3-dependent homodimerization of Bax or interactions with Bax activators such as Bid may either assist or be required for the cell death-inducing mechanism of this protein.

Bcl-2 family proteins play an important role as regulators of programmed cell death and apoptosis (1–3). Many Bcl-2 family proteins including the anti-apoptotic proteins Bcl-2 and Bcl-XL and the pro-apoptotic proteins Bax and Bak can physically interact with each other, forming a complex network of homo- and heterodimers (1, 2). The domains within these proteins required for dimerization have been determined by deletional and mutational analysis, and the results recently corroborated by x-ray crystallographic and NMR-based structural studies (4–14). The three-dimensional structure of the Bcl-XL protein consists of seven α-helices joined by flexible loops of variable length (13). Amino acid sequence alignments of Bcl-2 family proteins have demonstrated four evolutionarily conserved domains, termed Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4. The BH3 domain corresponds to the second amphipathic α-helix in these proteins, as predicted from the three-dimensional structure of Bcl-XL (13).

The BH1, BH2, and BH3 domains in combination form the borders of a hydrophobic pocket located on the surface of the Bcl-XL protein. Certain mutations that affect residues lining this pocket have been shown to abrogate their ability to dimerize with Bax (4, 9). Thus, this surface pocket appears to function analogous to a receptor, binding epitopes located on dimerizing partner proteins. Through a combination of deletional and mutagenesis studies (7, 8, 10–12, 15), peptide competition assays (16), and NMR-based structural analyses (14), it has been determined that the BH3 domain represents the counter-structure for dimerization which inserts similar to a peptide ligand into the surface pocket on Bcl-XL.

Bcl-2 family proteins therefore presumably exist in two conformations: one in which the protein creates a receptor-like pocket and the other in which the amphipathic α-helix that comprises BH3 rotates outward to expose its hydrophobic surface so that it can bury this side of the α-helix into the receptor-like pocket on dimerization partner proteins (14). Interestingly, some pro-apoptotic members of the Bcl-2 family, including Bik, Hrk, and Bid, contain only a BH3 domain (10–12). Deletion of the BH3 domains from these proteins uniformly abolishes their functions as inducers of cell death and antagonists of anti-apoptotic proteins such as Bcl-2 and Bcl-XL. Moreover, overexpression of fragments of the pro-apoptotic protein Bak which include only the BH3 domain but not the other BH domains has been shown to be sufficient for inducing apoptosis in mammalian cells (8).

In addition to providing insights into mechanism of dimerization, the three-dimensional structure of Bcl-XL has also revealed striking overall similarity to the pore-forming domains of certain bacterial toxins (13). Consistent with structural predictions, recombinant Bcl-2, Bcl-XL, and Bax proteins have been shown to form ion-conducting channels in liposomes...
and planar bilayers in vitro (17–19). Although the role of homo- and heterodimerization in pore formation remains to be explored, it seems likely that a minimum of two molecules is required to create an ion channel, based on structural considerations and comparisons with other α-helical-type channel proteins (18, 20, 21).

Despite the advances that have been made in understanding the structural aspects of dimerization among Bcl-2 family proteins, the functional significance of many of these protein-protein interactions remains controversial. For example, mutagenesis studies of the anti-apoptotic proteins Bcl-2 and Bcl-XL initially suggested that heterodimerization with the pro-apoptotic protein Bax was critical for their cell survival activities (4, 9). Subsequently, however, alanine substitution mutants of Bcl-XL were reported that failed to dimerize with Bax but which retained the ability to promote cell survival (22). Whether such heterodimerization-defective mutants of Bcl-XL could prevent cell death specifically induced by overexpression of Bax was not addressed, raising questions about whether heterodimerization might nevertheless be required for the mutual antagonism displayed by anti-apoptotic (Bcl-2/Bcl-XL) and pro-apoptotic (Bax) Bcl-2 family proteins.

The role of homo- and heterodimerization in Bax- and Bak-induced apoptosis has also generated controversy. For example, deletion of the BH3 domain from the Bax or Bak proteins as well as removal of four amino acids from within the BH3 of Bax comprising a well conserved IGDE motif was reported to abolish Bax/Bak-mediated cell death in mammalian cells (8, 15). These same BH3 domain mutations also abrogated the ability of Bax and Bak to induce cell death in yeast and negated homo- and heterodimerization (7, 8, 15, 23). However, when tested in another mammalian cell line, BaxΔBH3 was reported to accelerate apoptosis induced by chemotherapeutic drugs as effectively as wild-type Bax protein and to abrogate the cytoprotective effects of Bcl-XL overexpression, despite its inability to dimerize with either Bax or Bcl-XL (24, 25).

In this report, we performed an alanine-scanning mutagenesis analysis of the conserved IGDE motif within the BH3 domain of Bax, generating mutants of Bax which fail to heterodimerize with Bcl-2 but which retain their ability to homodimerize and to induce cell death in both yeast and mammalian cells. The findings derived from studies of these mutants indicate that Bax can promote cell death independently of heterodimerization with Bcl-2. Furthermore, evidence is presented that Bcl-2 can rescue both yeast and mammalian cells from cell death induced by overexpression of Bax, without binding to this pro-apoptotic protein. The implications of these findings with regards to mechanisms of Bcl-2 and Bax function are discussed.

MATERIALS AND METHODS

Plasmid Constructions—A murine bax cDNA (26) was employed as the template for mutagenesis experiments. Mutations were created using a two-step polymerase chain reaction method (7, 15). All mutants were initially subcloned between EcoRI and XhoI sites in the two-hybrid plasmid pEG202 in frame with the NH2-terminal LexA DNA-binding domain sequences (7, 15). The following mutagenic primers were used in combination with the wild-type Bax forward (5′-GGGAATTGGAGATGCACTGGATAGC-3′) and reverse (5′-CGAATTGGAGATGCACTGGATAGC-3′) primers: Bax(I/A), 5′-GGAGATGAACTG-3′ (forward) and 5′-CGAATTGGAGATGCACTGGATAGC-3′ (reverse); Bax(E/A), 5′-GTGGGGATGAACTGATGGTACG-3′ (forward) and 5′-CGAATTGGAGATGCACTGGATAGC-3′ (reverse); and Bax(G/A), 5′-GGAGATGAACTG-3′ (forward) and 5′-CGAATTGGAGATGCACTGGATAGC-3′ (reverse). These mutants were subsequently subcloned into pEG202 into pCDNA3-HA (27) by digestion with EcoRI and XhoI for studies in mammalian cells. Bax(I66A)ΔTM, Bax(G67A)ΔTM, Bax(D86A)ΔTM, and Bax(E69A)ΔTM were prepared using the same primers described above except substituting the wild-type Bax reverse primer for the ΔTM reverse primer 5′-CTCTCGAGCT-CACTGGCATGTTGGGCTCCGGA-A-3′ which introduces a stop codon just upstream of the TM domain. These mutants were subsequently subcloned from pEG202 into the Bax-E69A ΔTM expression vector pG4-5-based plasmid pG4-5-Bcl-2 (28) as one of the templates with mutagenic primers 5′-GAT-GCGTTCCACAAAGAGTCACCACCTGACCTGCGG-3′ (forward) and 5′-CCATTGGAGATGCACTGGATAGC-3′ (reverse), and murine Bax cDNA pSK-II-Bax (26) as the other template with mutagenic primers 5′-ATGGGATGGAAGATGGTGGACGAGTTATGC-3′ (forward) and 5′-CTTTGTGGTGAGGCAAGAT-3′ (reverse) in combination with the wild-type Bax forward and reverse primers described above. To create Bax(Bcl-2/BHI3), lacking the TM domain, the ΔTM reverse primer was used instead of wild-type Bax reverse primer. These chimeric cDNAs were subcloned into pEG202 into pCDNA3-HA by digestion with EcoRI and XhoI.

A cDNA encoding mouse Bid protein was obtained by polymerase chain reaction amplification from a mouse muscle cDNA library (CLONTECH, Inc.) using the following primers: 5′-GGGAATTGGCCTGTTAGGTAAGCCTGGAGCAACG-3′ (forward) and 5′-CTCTCGAGCT-CACTGGCATGTTGGGCTCCGGA-A-3′ (reverse). After digestion with EcoRI and XhoI, the resulting fragment was subcloned into pEG202, and then transferred to pG4-5, pFlag600, and pGEX4T1 by digestion with EcoRI and XhoI. The proper construction of all plasmids was confirmed by DNA sequencing.

Yeast Two-hybrid Assays—Protein-protein interactions were evaluated from yeast two-hybrid assay as described in detail previously, using either EGY48 cells for LEU2 reporter gene assays or EGY191 cells for lacZ reporter gene assays, in conjunction with pEG202 (LexA DNA-binding domain) and pG4-5 (B42 transactivation (TA) domain) plasmids (5, 7, 15, 29). Growth on leucine-deficient medium was scored 4 days after spotting on minimal medium plates containing 2% galactose, 1% raffinose to induce expression of the TA domain-containing proteins from the GAL1 promoter in pG4-5. Cells spotted on minimal medium glucose plates served as negative controls. Filter assays were similarly performed for β-galactosidase measurements, using cells plated on either galactose- or glucose containing minimal medium supplemented with 20 μg of ml/m. Colorimetric results were photographed after 0.5–2.0 h.

In Vitro Protein Binding Assays—

GST-Bid fusion protein was produced for in vitro binding assay by using bacteria (Promega, Inc., TNT lysates) and T7 RNA polymerase, as described (7). After washing extensively, SDS-polyacrylamide gel electrophoresis analysis and autoradiography were accomplished as described (7).

Yeast-based Cytotoxicity Assays—EGY191 cells were employed for analysis of effects of Bax mutants on cell viability. Transformations were performed by the lithium acetate method, using 1.5 μg of purified plasmid DNA (Bax and Bax mutants) and 5 μg of sheared, denatured salmon sperm (carrier) DNA. For Bcl-2 rescue experiments, the cells were additionally co-transformed with 2.5 μg of pG4-5-5-Bcl-2, pGJ4-5-Bax (Bcl-2/BHI3), or pG4-5. The cells were then plated on histidine-deficient (transformed with pEG202-based plasmids) or both histidine- and tryptophan-deficient (co-transformed with pEG202-based and pG4-5-based plasmids) minimal media supplemented with other essential amino acids. The plates were cultured at 30 °C for 4–5 days, and colonies were counted.

Mammalian Cell Apoptosis Assays—As described previously (15). 293 cells (8 × 10⁵) were cultured overnight in 60-mm diameter dishes in 3 ml of Dulbecco's modified Eagle's medium containing 10% horse serum (Duchefa Equine Serum, Tulare, CA). The cells were then transfected with 3 μg of various plasmids encoding mutant forms of Bax by a calcium phosphate precipitation method. To investigate the effects of Bcl-2 or Bid on cytotoxicity of Bax mutants, each of Bax mutant plasmids (3 μg) was co-transformed with 0–7.5 μg of pFlag600-Bid, pRc-CMV-Bcl-2, or the respective parental vectors without cDNA inserts. The transfection medium was replaced—8 h later with fresh pre-warmed Dulbecco's modified Eagle's medium, and the cells were
cultured for another ~24 h. The floating and adherent cells were then collected, pooled, and subjected to trypan blue exclusion assay or DAPI staining with viewing under a UV microscope.

Co-immunoprecipitation Assays—293 cells (2 × 10^6) were cultured overnight in 10 ml of Dulbecco's modified Eagle's medium containing 10% horse serum. The cells were then co-transfected with 10 μg of pRc-CMV-Bcl-2 and 10 μg each of pcDNA3-HA-Bax(G67A), pcDNA3-HA-Bax(D68A), pcDNA3-HA-Bax(E69A), and pcDNA3-HA-Bax(WT) or parental pcDNA3-HA vector by a calcium phosphate precipitation method. Approximately 60 h later, the cells were lysed in 0.3 ml of Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), and apropin (5 μg/ml). Immunoprecipitations were performed by incubation with 20 μl of protein G-Sepharose beads preabsorbed with 4 μg of anti-HA mouse monoclonal antibody (12CA5, Boehringer Mannheim) at 4 °C for 3 h. After 3 washes in 1.5 ml of lysis buffer, immune complexes were subjected to SDS-PAGE (14% gels) and immunoblot analysis using anti-HA, anti-human Bax, or anti-human Bcl-2 antiserum (30). Antibody detection was accomplished using biotinylated secondary antibodies and horseradish peroxidase-avidin followed by an enhanced chemiluminescence (ECL) system (Amersham) with exposure to x-ray film.

Immunoblot Assays—Yeast cells transformed with various plasmid DNAs were grown to an A₆₀₀ of 1–2. Cells lysates were prepared by the glass bead method and normalized for protein content (15). After SDS-PAGE (30 μg of protein/lane in 12% gels) and transfer to nitrocellulose, antigen detection was accomplished using anti-LexA rabbit antiserum as described (17, 15).

RESULTS

Generation of Bax Mutants That Fail to Heterodimerize with Bcl-2 but Retain Ability to Homodimerize—Previously we showed by deletional analysis that a well conserved motif, IGDE (residues 66–69), within the BH3 domain of the Bax protein is required for Bax homodimerization, heterodimerization with Bcl-2, and death promoting bioactivity in both yeast and mammalian cells (15). We therefore prepared alanine substitution mutants of each of these residues and tested them for ability to interact with themselves, wild-type Bax, or Bcl-2 in yeast two-hybrid assays where proteins are expressed with either a LexA DNA-binding domain or B42 trans-activation (TA) domain fused to their NH₂ termini. In addition, a mutant of Bax was prepared in which the BH3 domain of Bcl-2 was substituted for the BH3 domain of Bax. For these experiments, all proteins were expressed without the COOH-terminal membrane-anchoring domain (last ~20 amino acids), thus avoiding problems with targeting to the nucleus. The transmembrane (TM) domain is also required for Bax-induced killing in yeast (15), thus its removal is necessary for yeast two-hybrid protein-interaction assays. The expression of all of these proteins at comparable levels in yeast was confirmed by immunoblotting (not shown).

As summarized in Table I, the Bax(I66A) and Bax(G67A) proteins behaved similar to the wild-type Bax protein in yeast two-hybrid assays, retaining the ability to interact with wild-type Bax and Bcl-2 as well as to homodimerize with themselves. Although non-quantitative β-galactosidase filter assays were performed, no obvious differences were observed in the intensity of blue color produced by the Bax(I66A) and Bax(G67A) mutants compared with wild-type Bax. In contrast, the Bax(D68A) and Bax(E69A) mutants failed to interact with Bcl-2 but retained the ability to bind wild-type Bax and to homodimerize with themselves (Table I). Bax containing the BH3 domain of Bcl-2 was capable of interacting with wild-type Bax and Bcl-2 but did not homodimerize with itself. The specificity of these protein interactions detected by yeast two-hybrid assays was confirmed by use of various control plasmids lacking cDNA inserts or two-hybrid plasmids encoding irrelevant proteins such as Fas cytosolic domain (Table I and data not shown).

Some Bax BH3 Mutants Retain Cytotoxic Function in Yeast—To examine the effects of BH3 domain mutants on the death inducing activity of Bax in yeast, the same LexA-Bax fusion proteins were expressed with their COOH-terminal TM domains. Yeast transformed with the plasmids encoding wild-type Bax, Bax(I66A), Bax(G67A), and Bax(E69A) protein formed very few colonies when plated on histidine-deficient media and incubation for 4 days at 30 °C, the number of colonies was counted. Panels A and B were derived from separate experiments and are representative of a minimum of three experiments.

![Fig. 1. Bax(I66A), Bax(G67A), and Bax(E69A) retain cytotoxic activity in yeast. EGY191 yeast cells were transformed with 1.5 μg of pEG202-based plasmids (HIS3) encoding wild-type or mutant Bax proteins expressed as NH₂-terminal LexA fusion proteins with their TM domains under the control of an ADH1 promoter. After plating on histidine-deficient media and incubation for 4 days at 30 °C, the number of colonies was counted. Panels A and B were derived from separate experiments and are representative of a minimum of three experiments.](http://www.jbc.org/content/274/51/31484/F1)

![A](http://www.jbc.org/content/274/51/31484/F1a)

![B](http://www.jbc.org/content/274/51/31484/F1b)
>1,000/1 μg of DNA) were formed when yeast were transformed with plasmids encoding Bax(Bcl-2/BH3) and Bax(D68A) proteins or when control proteins such as lamin were expressed in these cells (Fig. 1, A and B). Consistent with our prior studies (15), a Bax(DIGDE) mutant in which the DIGDE motif (residues 66–69) was deleted also failed to inhibit colony formation when expressed in yeast. Thus, the Bax(166A), Bax(G67A), and Bax(E69A) mutants retain bioactivity in yeast, whereas the Bax(D68A) and Bax(Bcl-2/BH3) mutants do not.

Bcl-2 Abrogates Lethal Phenotype of Bax Mutants in Yeast—Bcl-2 can rescue yeast from the lethal effects of wild-type Bax protein (5, 15, 29). Because the Bax(E69A) mutant retained cytotoxic activity but failed to heterodimerize with Bcl-2, we wished to explore the effects of Bcl-2 on this mutant. For these experiments, the same plasmids were used for Fig. 1 which encode wild-type or mutant versions of Bax expressed as NH2-terminal HA epitope tags. Two days after transient transfection of 293T cells, cell lysates were prepared, normalizing for total protein content, and immunoprecipitated with anti-HA monoclonal antibody followed by SDS-PAGE/immunoblot analysis (Fig. 3). Bcl-2 co-immunoprecipitated with the HA-Bax(G67A) and wild-type Bax proteins, while the HA-Bax(D68A) and HA-Bax(Bcl-2/BH3) mutants were not detected.

The expression in yeast of all of the mutants of Bax tested here was confirmed by immunoblot analysis (Fig. 2, C and D). Since some of the Bax proteins were lethal, it was necessary to co-transform cells with a Bcl-2 expression plasmid, so that viable colonies of cells could be obtained for preparation of protein samples. All of the Bax, Bax mutant, and control proteins were expressed from the same pEG202 plasmid with NH2-terminal LexA DNA-binding domains, which functioned analogous to epitope tags for these studies and which also were intended to enhance the stability of certain mutant proteins. As shown in Fig. 2, C and D, incubation of blots with an anti-LexA antiserum revealed that all Bax mutants were produced at levels comparable to or in excess of the wild-type Bax protein. As might be expected, the mutants of Bax which failed to exhibit cytotoxic activity tended to accumulate to higher levels in yeast, suggesting that cells could tolerate higher levels of these proteins compared with wild-type Bax (Fig. 2).

Co-immunoprecipitation Analysis of Bax Mutants in Mammalian Cells Confirms Yeast Two-hybrid Results—Before testing the function of Bax mutants in mammalian cells, we first explored whether the protein interaction results observed in yeast two-hybrid experiments could be confirmed by co-immunoprecipitation assays where Bax mutants were co-expressed with Bcl-2 in human 293T epithelial cells. For these experiments, the wild-type and mutant Bax proteins were expressed with NH2-terminal HA epitope tags. Two days after transient transfection of 293T cells, cell lysates were prepared, normalized for total protein content, and immunoprecipitated with anti-HA monoclonal antibody followed by SDS-PAGE/immunoblot assay using anti-HA-2 antiserum. As shown in Fig. 3, Bcl-2 co-immunoprecipitated with the HA-Bax(G67A) and wild-type HA-Bax proteins. In contrast, Bcl-2 did not co-immunoprecipitate with the HA-Bax(D68A) protein and only poorly co-immunoprecipitated with the HA-Bax(E69A) protein (~5–10% of wild-type) (Fig. 3A). No Bcl-2 was detected in association with anti-HA immunoprecipitates prepared from cells transfected with a control plasmid pcDNA3-HA, thus confirming the specificity of the results. It was not possible to test the HA-Bax(166A) and HA-Bax(Bcl-2/BH3) mutants because these proteins were evidently unstable in mammalian cells and could not be detected.
In yeast two-hybrid assays, all of the Bax mutants created here retained the ability to bind to wild-type Bax. We therefore relied upon the endogenous expression of human Bax protein in 293T cells to evaluate the ability of our HA-tagged murine Bax mutants to co-immunoprecipitate with untagged endogenous Hu-Bax. Re-probing the same blot shown above with an anti-body specific for the Hu-Bax protein demonstrated that approximately equivalent amounts of Hu-Bax protein were associated with anti-HA immune complexes. Thus, the Bax(D67A), Bax(D68A), and Bax(E68A) mutants all retained the ability to bind to Bax in mammalian cells. Re-probing the same blot with anti-HA antibody confirmed production of nearly equal amounts of the HA-tagged wild-type and mutant Bax proteins. Taken together, these co-immunoprecipitation assays confirm the results of yeast two-hybrid experiments, indicating that the Bax(D67A) mutant retains the ability to dimerize with Bcl-2 and wild-type Bax, whereas the Bax(D68A) and Bax(E68A) mutants can bind to Bax but have little ability to bind Bcl-2.

Bax Promotes Apoptosis in Mammalian Cells Irrespective of Heterodimerization with Bcl-2—The apoptotic effects of these Bax mutants were compared with wild-type Bax by transient transfection in 293T cells, enumerating the percentage of cells stained with the DNA-binding fluorochrome DAPI. As shown in Fig. 4A, the wild-type Bax protein and the Bax(D67A), Bax(D68A), and Bax(E68A) mutant proteins induced 4–6-fold more apoptosis in 293T cells when compared with control (“NEO”) transfected cells. Thus, similar to the results obtained in yeast, the Bax(D67A) and Bax(E68A) protein promoted cell death in mammalian cells. However, unlike the results obtained in yeast, the Bax(D68A) protein was active in mammalian cells. These data indicate that Bax mutants which fail to heterodimerize efficiently with Bcl-2, i.e. Bax(D68A) and Bax(E68A), nevertheless retain their pro-apoptotic activity in mammalian cells.

Bcl-2 Abrogates Bax-induced Apoptosis in the Absence of Heterodimerization—To explore whether Bcl-2 could inhibit apoptosis induction by mutants of Bax which do not heterodimerize with it, 293T cells were co-transfected with equal amounts of Bax and Bcl-2 expression plasmids and the percentage of apoptotic cells was determined 1–1.5 days later by DAPI staining. As shown in Fig. 4B, Bcl-2 partially inhibited apoptosis induction by wild-type Bax (Bax(G67A), Bax(D68A), and Bax(E68A)). Immunoblot assay confirmed the production of Bcl-2 and showed that Bcl-2 did not reduce the amounts of wild-type and mutant Bax proteins, thus excluding this as a trivial explanation for the lower percentages of apoptotic cells (not shown).

To further examine the ability of Bcl-2 to abrogate cell death induction by mutants of Bax with impaired heterodimerization capacity, a fixed amount of wild-type Bax or Bax(E68A) expression plasmid (2.5 μg) was co-transfected with various amounts of Bcl-2 expression plasmid (0, 1.25, 2.5, and 7.5 μg). As shown in Fig. 4C, Bcl-2 inhibited cell death induction by the wild-type Bax and Bax(E68A) plasmids with the similar concentration dependence, indicating that the relative potency of Bcl-2 as a negator of Bax-induced apoptosis is unaffected by differences of heterodimerization efficiency. Similar results were obtained with the Bax(D68A) mutant protein (not shown). Taken together, these observations suggest that there is little or no relation between the ability of Bcl-2 to heterodimerize with Bax and its ability to protect cells from Bax-induced cell death.

Bax Mutants Retain Ability to Bind the Bid Protein and Collaborate with Bid to Induce Cell Death—The Bid protein is unique among the “BH3 only” subgroup of Bcl-2 family proteins in that it can heterodimerize with Bax and promote apoptosis
Heterodimerization-independent Functions of Bax and Bcl-2

How Bax promotes cell death in mammalian cells and yeast is controversial. At least two potentially independent mechanisms can be envisioned. First, the BH3 domain of Bax can bind to Bcl-2 and related anti-apoptotic proteins, thus potentially inactivating them. This mechanism is analogous to how the subgroup of pro-apoptotic Bcl-2 family proteins which contain only BH3 domains (e.g. Bik, Hrk) presumably functions (10, 11). Second, Bax may form cytotoxic channels for ions or other molecules in the intracellular membranes where it resides (19). We have speculated that the lethal phenotype displayed by some pro-apoptotic members of the Bcl-2 family such as Bax and Bak in budding and fission yeast is a manifestation of this channel activity, particularly since yeast contain no identifiable Bcl-2 homologs (15). Consistent with these ideas, it has been shown that expression of a fragment of Bak consisting essentially only of its BH3 domain tethered to a TM domain is sufficient for inducing apoptosis in mammalian cells in which BH3-binding anti-apoptotic proteins such as Bcl-2 and Bcl-XL are found, whereas such BH3 only mutants of Bak which lack the predicted pore-forming fifth and sixth α-helices are inactive in yeast (8, 23). In mammalian cells, the relative contributions of these two mechanisms for the pro-apoptotic functions of Bax and Bak remain to be clarified but cellular context seems likely to play a large role.

Based on the mutants of Bax characterized here, we surmise that BH3-dependent heterodimerization with Bcl-2 is not required for the pro-apoptotic function of Bax in mammalian cells. This was specifically shown by the ability of the Bax(D68A) and Bax(E69A) mutants to induce apoptosis when overexpressed in 293T cells, without forming heterodimers with Bcl-2. Although it is difficult to exclude the possibility that these mutants of Bax retain the ability to heterodimerize with other anti-apoptotic members of the Bcl-2 family that may be present in mammalian cells, these mutants also do not interact with Bcl-X\(_L\) (data not presented). Thus, Bax apparently need not heterodimerize with Bcl-2 or Bcl-X\(_L\) to promote apoptosis in mammalian cells. Moreover, the observation that Bcl-2 can protect human cells from cell death induced by overexpression of the Bax(D68A) and Bax(E69A) mutants implies that it is unnecessary for Bcl-2 to dimerize with Bax for negating apoptosis induced by overexpression of this protein. Similar results have been obtained by Simonian et al. (24, 25) using a double alanine substitution mutant of Bax in which Asp-68 and Glu-69 were simultaneously converted to alanine, showing a failure of this protein to bind Bcl-X\(_L\) while still retaining its ability to accelerate cell death induced by chemotherapeutic drugs and to negate the cytotoxic effects of Bcl-X\(_L\). However, those and other studies (22) did not explore whether apoptosis induced specifically by overexpression of Bax could also be inhibited by Bcl-X\(_L\) through a heterodimerization-independent mechanism. Another report by Tao et al. (31) similarly found that mutants of Bcl-X\(_L\), with reduced ability to heterodimerize with Bax, retained active as suppressors of Bax-induced cell death in yeast, also supporting the idea of heterodimerization-independent antagonism of Bax. However, those Bcl-X\(_L\) mutants were only partially defective in Bax-binding, thus limiting interpretation of the results (31).

Previously we showed that deletion of the IGDE motif in the BH3 domain of Bax or removal of the entire BH3 domain prevented these proteins from homodimerizing and from inducing cell death in yeast and mammalian cells (7, 15). Although some other studies have explored the effects of BH3 domain mutations on the ability of Bax and Bak to interact with Bcl-X\(_L\) or to bind the wild-type Bax protein (8, 24, 25), they have not addressed the issue of whether these mutant Bax proteins...
could still homodimerize with themselves and whether this related to their function as death-inducing molecules. None of the alanine-substitution mutants of the IGDE motif within Bax BH3 domain interfered with homodimerization, as determined by yeast two-hybrid assays. In human 293T cells, these homodimerization-competent mutants of Bax also retained the ability to induce apoptosis, implying that homodimerization and cell death may be correlated. In addition, the Bax(Bcl-2/BH3) protein, in which the BH3 domain from Bcl-2 was substituted for that of Bax, failed to homodimerize and was inactive at inducing cell death in yeast, again suggesting the idea that homodimerization may be correlated with Bax bioactivity. Unfortunately, this chimeric Bax(Bcl-2/BH3) protein was unstable in mammalian cells, precluding testing in that context. However, one of the mutants, Bax(D68A), lost its cell death activity in yeast, and yet remained capable of homodimerizing with itself. Thus, if homodimerization is required for the function of Bax, it evidently can be insufficient. Why the Bax(D68A) mutant was fully active in mammalian cells but impaired in its function in yeast remains to be determined, but presumably can be attributed to the greater complexity of Bcl-2/Bax family protein regulation and function in mammalian cells, where a variety of dimerizing homologs and other interacting proteins as well as post-translational modifications can occur that are not found in yeast (3, 21).

It has been shown that Bcl-2 can prevent Bax-channel formation in synthetic lipid membranes in vitro (19). Several mechanisms by which Bcl-2 might interfere with Bax channel formation can be envisioned. For example, BH3-dependent heterodimerization of Bcl-2 with Bax could prevent Bax from dimerizing with Bcl-2 in vitro, as well as post-translational modifications can occur that are not found in yeast (3, 21).

The Bid protein is unique among the BH3-only subgroup of pro-apoptotic Bcl-2 family proteins in that it binds not only to anti-apoptotic members of the family such as Bcl-2 and Bcl-XL, but also interacts with Bax (12). Like other BH3-only Bcl-2 family proteins, however, Bid is incapable of homodimerizing with itself. Preliminary mutagenesis studies of the BH3 domain of Bid suggest that binding to Bax rather than to Bcl-2 or Bcl-XL correlates with its pro-apoptotic activity in mammalian cells (12). If confirmed by further analysis, this observation suggests that some BH3-dependent interactions with Bax may enhance its lethal function, we presume by somehow promoting Bax channel formation since the binding of the BH3 domain of Bid to Bax should preclude Bax from dimerizing with Bcl-2.

Bcl-XL and acting as a trans-dominant inhibitor of these anti-apoptotic Bcl-2 family proteins (16). By analogy, the BH3-dependent homodimerization of Bax with itself may similarly play a role in the function of this protein as a promoter of cell death. Thus, we favor the notion that BH3-dependent homodimerization may facilitate or even be required for channel formation by Bax, inasmuch as removal of this domain from Bax (and Bak) abolishes its cytotoxic function in yeast (15, 23). However, until these ideas have been confirmed directly by studies of Bax channels in vitro and such channels have been demonstrated in vivo, they should be viewed only as speculations.

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