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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(ΔIGDE), 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-X_L 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-X_L 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 up to four evolutionarily conserved domains, termed Bcl-2 homology (BH)[1] 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-X_L (13).

The BH1, BH2, and BH3 domains in combination form the borders of a hydrophobic pocket located on the surface of the Bcl-X_L 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 counterstructure for dimerization which inserts similar to a peptide ligand into the surface pocket on Bcl-X_L.

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-X_L. 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-X_L has also revealed striking overall similarity to the pore-forming domains of certain bacterial toxins (13). Consistent with structural predictions, recombinant Bcl-2, Bcl-X_L, and Bax proteins have been shown to form ion-conducting channels in liposomes

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[1] The abbreviations used are: BH, Bcl-2 homology; GST, glutathione *S*-transferase; TM, transmembrane; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole.

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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-X_L 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-X_L 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-X_L 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-X_L) 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, Bax(ΔBH3) was reported to accelerate apoptosis induced by chemotherapeutic drugs as effectively as wild-type Bax protein and to abrogate the cytoprotective effects of Bcl-X_L overexpression, despite its inability to dimerize with either Bax or Bcl-X_L (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 *Eco*RI and *Xho*I sites in the two-hybrid plasmid pEG202 in frame with the NH_2-terminal LexA DNA-binding domain sequences (7, 15). The following mutagenic primers were used in combination with the wild-type Bax forward (5′-GGGAATTCGCGGTGATGGACGGGT-3′) and reverse (5′-GTCTCCGGCGAGCTGGAGATGAACTG-3′) primers: Bax(I/A), 5′-GTCTCCGDCGAGCTGGAGATGAACTG-3′ (forward) and 5′-CAGTTCATCTCCAGCTCGCCGGAGAC-3′ (reverse); Bax(G/A), 5′-TCCGGCGAATTGCAGATGAACTGG-3′ (forward) and 5′-CCAGTTCATCTGCAATTCGCCGAG-3′ (reverse); Bax(D/A), 5′-GCGAATTGGAGCTGAACTGGATAG-3′ (forward) and 5′-CTATCCAGTTCAGCTCCAATTCGC-3′ (reverse); Bax(E/A), 5′-CGAATTGGAGATGCACTGGATAGC-3′ (forward) and 5′-GCTATCCAGTGCATCTCCAATTCG-3′ (reverse). These mutants were subsequently subcloned from pEG202 into pcDNA3-HA (27) by digestion with *Eco*RI and *Xho*I for studies in mammalian cells. Bax(I66A)ΔTM, Bax(G67A)ΔTM, Bax(D68A)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′-CTCTCGAGTCACTGCCATGTGGGGGTCCCGAA-3′ which introduces a stop codon just upstream of the TM domain. These mutants were subsequently subcloned from pEG202 into the B42 trans-activation domain-encoding yeast two-hybrid plasmid pJG4-5 by digestion with *Eco*RI and *Xho*I.

Bax(Bcl-2/BH3), a cDNA encoding Bax in which the BH3 domain (residues 59–73) was replaced with the BH3 domain of human Bcl-2 (residues 93–107), was generated using the human Bcl-2 cDNA pSKII-Bcl-2 (28) as one of the templates with mutagenic primers 5′-GATGCGTCCACCAAGAAGGTCCACCTGACCCTCCGCAG-3′ (forward) and 5′-CATCCTCTGCAGCTCCATGCGGCGGTAGCGGCGGGAG-3′ (reverse), and murine Bax cDNA pSK-II-Bax (26) as the other template with mutagenic primers 5′-ATGGAGCTGCAGAGGATGATTGC-3′ (forward) and 5′-CTTCTTGGTGGA CGCATCCTGG-3′ (reverse) in combination with the wild-type Bax forward and reverse primers described above. To create Bax(Bcl-2/BH3) lacking the TM domain, the ΔTM reverse primer was used instead of wild-type Bax reverse primer. These chimeric cDNAs were subcloned from pEG202 into pcDNA3-HA by digestion with *Eco*RI and *Xho*I.

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′-GGGAATTCGCGGTGATGGACTCTGAGGTCAGCAACG-3′ (forward) and 5′-CTCTCGAGTCAGTCCATCTCGTTTCTAACCAAG-3′ (reverse). After digestion with *Eco*RI and *Xho*I, the resulting fragment was subcloned into pEG202, and then transferred to pJG4-5, pFlag600, and pGEX4T1 by digestion with *Eco*RI and *Xho*I. The proper construction of all plasmids was confirmed by DNA sequencing.

Yeast Two-hybrid Assays—Protein-protein interactions were evaluated by 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 pJG4-5 (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 pJG4-5. 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 leucine/ml. Colorimetric results were photographed after 0.5–2.0 h.

In Vitro Protein Binding Assays—GST-Bid fusion protein was produced in BL21 stain cells after induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4–6 h at room temperature and purified on glutathione-Sepharose. The fusion protein (5 μg) was immobilized on glutathione-Sepharose and incubated with 0.01 ml of *in vitro* translated, L-[^{35}S]methionine-labeled proteins ((Bax(G67A), Bax(D68A), Bax(E69A), and Bax(WT)), which were prepared from pcDNA3-HA templates using a coupled transcription/translation system employing rabbit reticulocytes lysates (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 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 pJG4-5-Bcl-2, pJG4-5-Bax-(Bcl-2/BH3), or pJG4-5. 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 pJG4-5-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 (8×10^5) were cultured overnight in 60-mm diameter dishes in 3 ml of Dulbecco's modified Eagle's medium containing 10% horse serum (Donor Equine Serum, Tulare, CA). The cells were then transfected with 3 μg of various plasmids encoding wild-type or mutants 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

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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-4) containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), and aprotinin (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 $A_{600\ nm}$ 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 (7, 15).

TABLE I
Yeast two-hybrid analysis of Bax BH3 domain mutants

Two-hybrid assays were performed using EGY191 cells transformed with plasmids encoding the indicated LexA DNA-binding domain or B42 trans-activation (TA) domain fusion proteins and plated on galactose-containing medium to induce expression of the *GAL*1 promoter in the pJG4–5. In all cases, Bax and Bcl-2 proteins lacked the membrane-anchoring TM domain. The results of β-galactosidase (β-Gal) filter assays are presented, where development of a clear blue color within 2 h is denoted by "+." In controls where cells were plated on glucose-containing medium, negligible color development was observed (not shown).

LexA		TA	β-Gal
Bax(I66A)	×	Bax(I/A)	+
Bax(I66A)	×	Bax	+
Bax(I66A)	×	Bcl-2	+
Bax(I66A)	×	Fas	−
Bax(G67A)	×	Bax(G/A)	+
Bax(G67A)	×	Bax	+
Bax(G67A)	×	Bcl-2	+
Bax(G67A)	×	Fas	−
Bax(D68A)	×	Bax(D/A)	+
Bax(D68A)	×	Bax	+
Bax(D68A)	×	Bcl-2	−
Bax(D68A)	×	Fas	−
Bax(E69A)	×	Bax(E/A)	+
Bax(E69A)	×	Bax	+
Bax(E69A)	×	Bcl-2	−
Bax(E69A)	×	Fas	−
Bax(Bcl-2/BH3)	×	Bax(Bcl-2/BH3)	−
Bax(Bcl-2/BH3)	×	Bax	+
Bax(Bcl-2/BH3)	×	Bcl-2	+
Bax(Bcl-2/BH3)	×	Fas	−

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 NH_2 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 used 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 for selection of the pEG202 plasmid encoding these proteins (Fig. 1*A*). In contrast, numerous colonies (typically

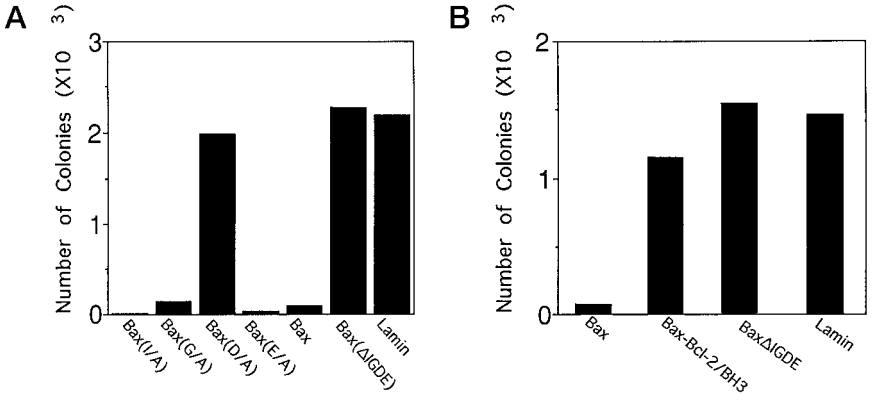

FIG. 1. **Bax(I66A), Bax(G67A), and Bax(E69A) retain cytotoxic activity in yeast.** EGY191 yeast cells were transformed with 1.5 μg of pEG202-based plasmids (*HIS3*) encoding wild-type or mutant Bax proteins expressed as NH_2-terminal LexA fusion proteins with their TM domains under the control of an *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.

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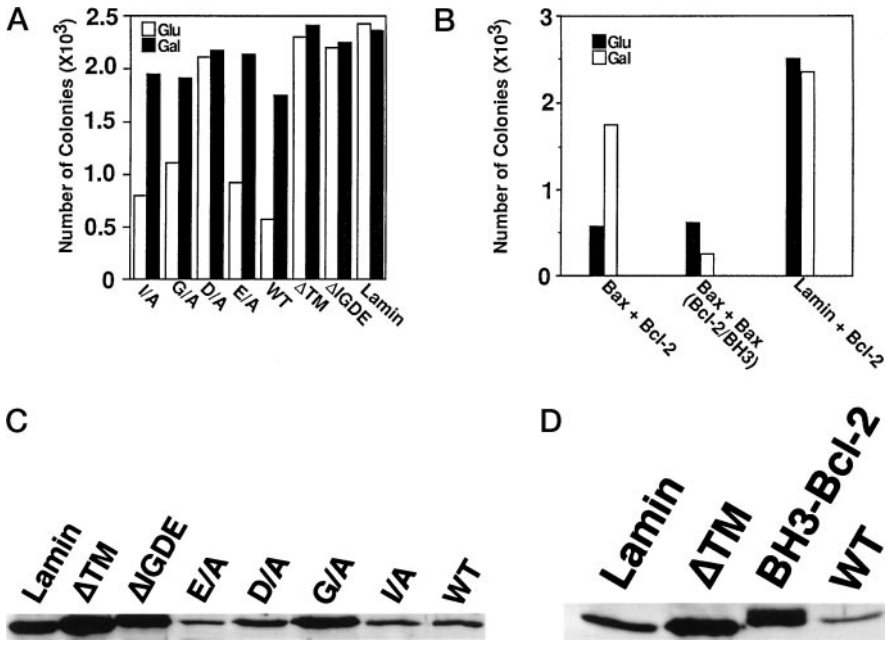

FIG. 2. **Bcl-2 rescues yeast from cell death induced by heterodimerization-defective Bax(E69A) protein.** *A* and *B*, EGY191 cells were co-transformed with 2.5 μg of pJG4-5-Bcl-2 or pJG4-5-Bax (Bcl-2/BH3) plasmids and 1.5 μg of pEG202-Bax or various mutants pEG202-Bax mutants as indicated. The cells were then plated on either glucose- or galactose-containing media to induce the *GAL1* promoter in pJG4-5, without histidine and tryptophan to select for the pEG202 and pJG4-5 plasmids, respectively. After 4–5 days incubation at 30 °C, the number of colonies was counted. *C* and *D*, the relative levels of the LexA-fusion proteins were examined by immunoblot analysis of 30 μg of total protein lysate, using an anti-LexA antiserum.

>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(ΔIGDE) mutant in which the IGDE motif (residues 66–69) was deleted also failed to inhibit colony formation when expressed in yeast. Thus, the Bax(I66A), 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 NH_2-terminal LexA fusions with their TM domains were employed, performing co-transformations with a Bcl-2 expression plasmid, pJG4-5-Bcl-2. Colony formation on selective medium was then scored 4–5 days later. As expected by their ability to heterodimerize with Bcl-2, colony formation by yeast co-transformed with Bcl-2 and wild-type Bax, Bax(I66A), or Bax(G66A) was not inhibited compared with control cells which had been transformed with the parental plasmids (not shown) or irrelevant plasmids such as pEG202-lamin (Fig. 2*A*). In addition, however, Bcl-2 also rescued yeast expressing the Bax(E69A) mutant (Fig. 2*A*), which fails to interact with Bcl-2 in yeast two-hybrid experiments (Table I). The specificity of these results was confirmed by use of additional mutants of Bax including Bax(ΔIGDE) and Bax(ΔTM), the latter of which is missing the COOH-terminal membrane anchoring domain required for cytotoxic activity in yeast (15). These observations, therefore, suggest that heterodimerization is not required for Bcl-2-mediated suppression of Bax in yeast.

The Bax(Bcl-2/BH3) mutant failed to kill yeast, but according to yeast two-hybrid experiments, can still bind to wild-type Bax. It was possible therefore that this chimeric protein which contains the BH3 domain of Bcl-2 might function akin to Bcl-2, forming a heterodimer with Bax and suppressing its cytotoxic activity in yeast. However, when co-expressed with wild-type Bax in yeast, the Bax(Bcl-2/BH3) protein failed to rescue (Fig. 2*B*). These data argue, therefore, that heterodimerization is apparently insufficient for nullifying the lethal effects of Bax in yeast.

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 NH_2-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 NH_2-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-Bcl-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. 3*A*). 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(I66A) and HA-Bax(Bcl-2/BH3) mutants because these proteins were evidently unstable in mammalian cells and could not be detected.

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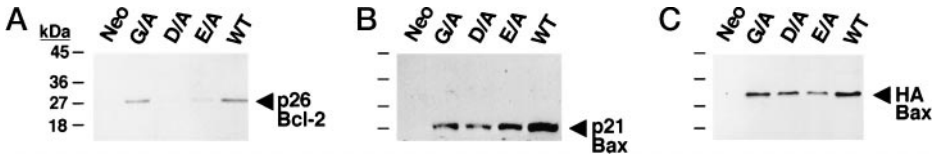

FIG. 3. **Bax(D68A) and Bax(E69A) exhibit little or no ability to co-immunoprecipitate with Bcl-2 in mammalian cells.** Wild-type or mutants of Bax were expressed as HA epitope-tagged proteins using pcDNA3-HA plasmids. The 293 cells were co-transfected with 10 μg of pRcCMV-Bcl-2 and 10 μg of each of these Bax mutants by a calcium phosphate precipitation method. Approximately 60 h later, cell lysates were prepared. After normalization for total protein content, lysates were either immunoprecipitated (300 μg) with anti-HA monoclonal antibody (*A* and *B*) or applied directly to gels (20 μg) (*C*) and then analyzed by SDS-PAGE immunoblotting using anti-human Bcl-2 (*A*), anti-human Bax (*B*), or anti-HA (*C*) antibodies.

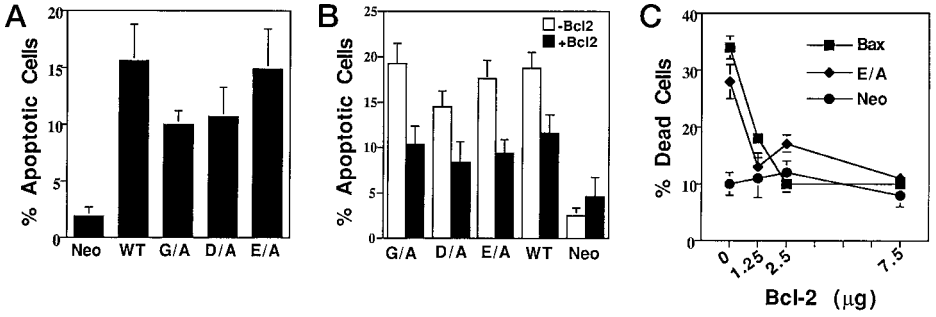

FIG. 4. **Apoptosis induced by Bax(G67A), Bax(D68A), and Bax(E69A) in human 293 epithelial cells can be abrogated by Bcl-2.** *A*, 293 cells were transfected with 3 μg of pcDNA3-HA-Bax mutant plasmids or parental pcDNA3-HA vector ("*Neo*") by a calcium phosphate precipitation method. Approximately 36 h later, the floating and adherent cells were pooled and stained with DAPI, and the percentage of cells with apoptotic nuclear morphology was determined (mean ± S.D.; $n = 3$). *B*, 293 cells were co-transfected with 3 μg of either pRc-CMV-Bcl-2 (*dark bars*) or pRc-CMV control plasmid (*white bars*) and 3 μg of various pcDNA-HA-Bax plasmids as indicated. The percentages of apoptotic cells were enumerated by DAPI-staining after ~36 h (mean ± S.D.; $n = 3$). *C*, 293 cells were co-transfected with 3 μg of pcDNA3-HA-Bax plasmids and 0–7.5 μg of pRc-CMV-Bcl-2, using pcDNA3-HA to maintain a fixed total amount of DNA. Floating and adherent cells were pooled ~36 h later and the percentage of dead cells was determined by trypan blue positivity (mean ± S.D.; $n = 3$). In all cases, transfection efficiencies were >80% based on parallel co-transfections performed with 1 μg of pCMV-β-gal or pGFP-N2 (CLONTECH) plasmid DNA admixed with an appropriate amount of pcDNA3-HA to normalize for total DNA content (not shown).

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 antibody 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(G67A), 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(G67A) mutant retains the ability to dimerize with Bcl-2 and wild-type Bax, whereas the Bax(D68A) and Bax(E69A) 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 with nuclear fragmentation and chromatin condensation by staining with the DNA-binding fluorochrome DAPI. As shown in Fig. 4*A*, the wild-type Bax protein and the Bax(G67A), Bax(D68A), and Bax(E69A) 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(G67A) and Bax(E69A) 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(E69A), 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 ~1.5 days later by DAPI staining. As shown in Fig. 4*B*, Bcl-2 partially inhibited apoptosis induced 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 is 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(E69A) 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 Fig. 4*C*, 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 (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

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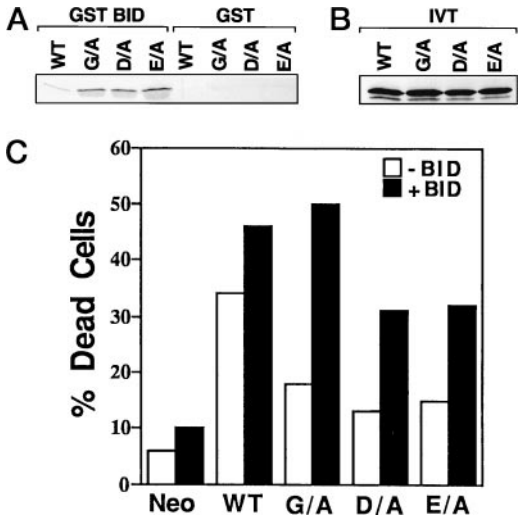

FIG. 5. **Bax mutants, Bax(G67A), Bax(D68A), and Bax(E69A) heterodimerize with Bid and collaborate with Bid in apoptosis induction.** GST-Bid fusion protein and GST control protein (5 μg) immobilized on glutathione-Sepharose (10 μl) were tested for binding to ^{35}S-labeled *in vitro* translated proteins Bax and mutant Bax proteins. *A*, proteins that associated with GST fusions were analyzed by SDS-PAGE (12%) and radiofluorography. *B*, as a control, 1 μl of reticulocyte lysates containing *in vitro* translated proteins was run directly in gels. *C*, 293 cells were co-transfected with 3 μg of pcDNA3-HA-Bax wild-type or mutant plasmids and either 3 μg of pFlag600-BID (*dark bars*) or pFlag600 (*white bars*) by a calcium phosphate precipitation method. The floating and adherent cells were pooled ~36 h later and analyzed by trypan blue dye exclusion assay. Transfection efficiencies were >80% for all samples, as determined by co-transfection with 1 μg of pGFP-N2.

(12). Site-specific mutagenesis of the BH3 domain of Bid has suggested that its ability to heterodimerize with Bax rather than with Bcl-2 or Bcl-X_L correlates with Bid-induced apoptosis (12). We therefore explored whether the Bax mutants described here could bind to Bid and collaborate with Bid in inducing apoptosis.

For protein-binding experiments, a GST-Bid fusion protein was produced in bacteria, affinity-purified on glutathione-Sepharose, and tested for binding to *in vitro* translated HA-tagged wild-type Bax and Bax mutant proteins. As shown in Fig. 5*A*, the mutant Bax(G67A), Bax(D68A), and Bax(E69A) proteins bound to GST-Bid at least as efficiently as wild-type Bax, indicating that they retain full Bid binding activity. Indeed, these mutants of Bax consistently appeared to bind better to GST-Bid than wild-type Bax ($n = 3$). In contrast, none of these Bax proteins interacted with GST control protein, confirming the specificity of the results.

Next, 293T cells were transiently co-transfected with Bid and Bax mutants, using a concentration of Bid-encoding plasmid that was insufficient by itself to induce significant amounts of cell death. When co-transfected with either wild-type Bax or the mutants of Bax, Bid increased the percentage of dead cells, suggesting that Bid can enhance the apoptotic activity of Bax and the Bax mutants examined here. Immunoblotting confirmed production of the Flag-Bid and HA-Bax proteins and showed that Bid does not increase the levels of HA-Bax or HA-Bax mutant proteins in 293T cells (not shown). Thus, while the Bax(D68A) and Bax(E69A) mutants exhibit little or no Bcl-2 binding activity, they retain the ability to bind and collaborate functionally with Bid. These data therefore argue that the alanine substitution mutations created within Bax have not grossly altered the function of these proteins or their general ability to interact with other proteins.

DISCUSSION

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-X_L 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 cytoprotective 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 remained 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

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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 supporting 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 undergoing the conformational changes necessary for integration of its α5- and α6-helices into membranes, which represents the first step in channel formation. To the extent that Bax-mediated killing of yeast is a surrogate assay for Bax-channel activity, our data with the Bax(E68A) mutant which fails to dimerize with Bcl-2 discount this mechanism, since Bcl-2 was still able to rescue yeast from Bax(E69A)-induced cell death. Alternatively, or in addition, once Bax has integrated into membranes with its fifth and sixth α-helices penetrating perpendicularly through the lipid bilayer, Bcl-2 could possibly then interfere with the assembly of two or more Bax molecules in the membrane during a second step of channel formation where pairs of α-helices come together to form a ring which creates an aqueous lumen for transport of ions of other molecules. It should be noted that protein-protein interactions of the type suggested above involving the membrane-penetrating α5- and α6-helices may not be detectable by routine co-immunoprecipitation assays and most likely would not be discerned by yeast two-hybrid methods.

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-X_L, 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-X_L 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-X_L 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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