Cytoprotection by Bcl-2 Requires the Pore-forming α5 and α6 Helices*

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We explored whether the putative channel-forming fifth and sixth α-helices of Bcl-2 and Bax account for Bcl-2-mediated cell survival and Bax-induced cell death in mammalian cells and in the yeast Saccharomyces cerevisiae. When α5-α6 were either deleted or swapped with each other, the Bcl-2α5α6 deletion mutant and Bcl-2-Bax(α5α6) chimeric protein failed to block apoptosis induced by either Bax or staurosporine in human cells and were unable to prevent Bax-induced cell death in yeast, implying that the α5-α6 region of Bcl-2 is essential for its cytoprotective function. Additional experiments indicated that, although α5-α6 is necessary, it is also insufficient for the anti-apoptotic activity of Bcl-2. In contrast, deletion or substitution of α5-α6 in Bax reduced but did not abrogate apoptosis induction in human cells, whereas it did completely nullify cytotoxic activity in yeast, implying that the pore-forming segments of Bax are critical for conferring a lethal phenotype in yeast but not necessarily in human cells. Baxα5α6 and Bax-Bcl(2α5α6) also retained the ability to dimerize with Bcl-2. Bax therefore may have redundant mechanisms for inducing apoptosis in mammalian cells, based on its ability to form α5-α6-dependent channels in membranes and to dimerize with and antagonize anti-apoptotic proteins such as Bcl-2.

Bcl-2 family proteins play a pivotal role in the regulation of programmed cell death and apoptosis. Some members of this family such as Bcl-2 and Bcl-XL function as cell death suppressors, whereas others such as Bax and Bak induce apoptosis (1–3). At least three biochemical characteristics have been ascribed to various Bcl-2 family proteins, including: (a) dimerization with themselves and each other; (b) interactions with other types of proteins, ranging from protein kinases and phosphatases to proteins that bind cell death proteases of the caspase family; and (c) formation of pores or ion channels in membranes (1). The relative significance of these different functions remains to be clarified, but may depend on the precise repertoire of Bcl-2 family proteins expressed in cells and the type of cell death stimuli applied.

The three-dimensional structure of one of the Bcl-2 family proteins, Bcl-XL, has been determined, revealing seven α-helices separated by flexible loops (4). Some other members of the Bcl-2 family, including the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax, can be readily modeled on the Bcl-XL crystallographic coordinates, implying that they share a similar fold despite having opposing effects on cell life and death (5). The C terminus of many Bcl-2 family proteins consists of a stretch of hydrophobic amino acids that serves the purpose of anchoring them within intracellular membranes, particularly the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope, with the bulk of the protein oriented toward the cytosol (6, 7).

Comparisons with other proteins for which structures are available revealed striking structural similarity of Bcl-XL to the pore-forming domains of certain bacterial toxins, including: (a) diphtheria toxin, which produces pores for transporting a polypeptide fragment of the toxin across lysosomal/endosomal membranes into the cytosol (8, 9); and (b) the colicins, which form ion channels that kill sensitive Escherichia coli by depolarizing their inner membranes (10). Moreover, Bcl-2, Bcl-XL, and Bax have been reported to form ion channels in synthetic membranes in vitro, when tested under conditions similar to those required for channel formation by diphtheria toxin or the colicins (11–14). However, the characteristics of the channels formed in vitro by cytoprotecive (Bcl-2, Bcl-XL) and cytotoxic (Bax) members of the Bcl-2 family differ. In general, Bcl-2 and Bcl-XL tend to form channels having low conductance, displaying modest cation selectivity, and exist in a mostly closed state, whereas Bax channels typically have 100–1000-fold larger conductances than Bcl-2 or Bcl-XL channels, prefer anions, and dwell longer in an open state (reviewed in Ref. 5).

By analogy to structurally similar pore-forming domains from bacterial toxins, the predicted fifth and sixth α-helices of Bcl-2 and Bax are hypothesized to directly participate in channel formation. These α-helices are positioned in the core of these proteins (based on models derived from the Bcl-XL structure) and are believed to be inserted into the membrane bilayer perpendicular to the membrane surface, with the loop connecting α5 and α6 presumably protruding from the other side of the membrane (5). Indeed, deletion of the α5-α6 regions from Bcl-2 abolishes its ability to form ion channels in synthetic membranes in vitro (12). The structural basis for differences in the channels formed in vitro by Bcl-2 and Bax is unknown, but could be due at least in part to differences between the polar residues of the fifth and sixth α-helices of these proteins. Two acidic amino acids are predicted to be on the hydrophilic face of α5 in Bcl-2 and Bcl-XL, which would presumably line the lumen channel, compared with two basic amino acids in the corresponding position for the pro-apoptotic Bax and Bak proteins (reviewed in Ref. 5). These differences in α5 and α6 might account for the relative cation specificity of the Bcl-2 and Bcl-XL channels (11, 12), and the anion selectivity of the Bax channel (13).

It remains to be determined whether channels are formed by Bcl-2 family proteins in vivo and whether this activity is critical for the biological functions of these proteins. However,
intrinsic bioactivities for the Bcl-2 and Bax proteins have been demonstrated in yeast, where no Bcl-2 homologs apparently exist based on sequence homology searches of the now completed genome of Saccharomyces cerevisiae. The Bax and Bak proteins, for example, confer a lethal phenotype when ectopically expressed in either the budding yeast S. cerevisiae or the fission yeast Schizosaccharomyces pombe (15–21). In contrast, mutants of Bax and Bak that lack the putative pore-forming a5 and a6 helices are devoid of cytotoxic activity in yeast. Bcl-2 and Bcl-XL can rescue yeast from the lethal effects of Bax and Bak, without necessity for dimerization between these proteins (22). Moreover, ectopic expression of Bcl-2 in the absence of Bax or Bak in certain mutant strains of yeast has also been shown to preserve cell viability under some circumstances (23), providing further evidence of an intrinsic function for this anti-apoptotic protein.

In this report, we explored some of the structure-function relations of the Bcl-2 and Bax proteins that may be relevant to their similarity to pore-forming proteins, focusing specifically on the putative pore-forming a5 and a6 helices. The results provide further insights into the question of why Bcl-2 is cytoprotective and Bax is cytodestructive, and suggest that differences in the a5 and a6 helices of Bcl-2 and Bax are necessary but insufficient for determining the opposing phenotypes of the proteins.

**MATERIALS AND METHODS**

**Plasmid Constructions**—Human Bcl-2 and human Bax cDNAs were employed as the templates for the mutagenesis experiments. Mutations were created using a two-step polymerase chain reaction method (17, 24). All mutants were initially subcloned between EcoRI (5’ end) and Xhol (3’ end) sites in pEG202, pJG4–5, pcDNA3, or pcDNA-HA plasmids. The following mutagenic primers were used in combination with the wild-type Bcl-2 forward (for pEG202, pJG4–5; 5’-GGCGAATTC-TGGCCGCACTCTGGAGAAC-3’; for pcDNA3, 5’-GGCGAATTC-TGGCCGCACTCTGGAGAAC-3’) and reverse (with C-terminal transmembrane domain (TM): 5’-ATTCCTGACATCTGTTGCCCCAGATAGCC-3’; without TM: 5’-CGCTCTGAGTCATGCTCTGCAGAGCAGGAC-3’) for the wild-type Bcl-2 (forward for pEG202, pJG4–5, or pcDNA-HA: 5’-GGCGAATTTGGACGGGCTGGGAGGAGG-3’; for pcDNA3: 5’-GGCGAATTTGGACGGGCTGGGAGGAGG-3’) and reverse (with C-terminal transmembrane domain (TM): 5’-ATTCCTGAGTTGCCGTCAGAGATGCC-3’; without TM: 5’-ATTCCTGAGTTGCCGTCAGAGATGCC-3’) sites in pEG202, pJG4–5, pcDNA3, or pcDNA-HA. For the construction of Bcl-2-Bax or Bax-Bcl-2 chimeras, first a SacI site was introduced into the Bax cDNA by two-step polymerase chain reaction using 5’-GGCGAATTC-TGGCCGCACTCTGGAGAAC-3’ (forward) and 5’-GGCGGCTGGGTTGGGGACCGGAGAAGG-3’ (reverse) or 5’-GGCGAATTC-TGGCCGCACTCTGGAGAAC-3’ (forward) and 5’-GGCGGCTGGGTTGGGGACCGGAGAAGG-3’ (reverse) with the above primers for wild-type Bax. Then, the region of the Bcl-2 cDNA and Bax cDNAs between the SacI and BamHI sites was swapped. For the production of recombinant GST-Bax (ΔTM) and GST-BaxΔa5a6 (ΔTM), cDNAs encoding GST (ΔTM) and BaxΔa5a6 (ΔTM) were subcloned between EcoRI (5’ end) and Xhol (3’ end) sites in pcDNA-Δ1-1 vector.

**Mammalian Cell Apoptosis Assays**—293T cells were cultured for 12 h in 60-mm diameter dishes in 5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Fresh medium was exchanged, and 4 h later the cells were co-transfected with 0.5 μg of pEGFP (CLONTECH Laboratories, Inc.) and various plasmids encoding wild-type or mutants of Bcl-2 or Bax by a calcium phosphate precipitation method (total amount of DNA normalized to either 1.5 or 2.5 μg). Four hours after transfections, fresh medium was exchanged and the cells were cultured for another 20 h before collecting both floating and adherent cells. Half of the recovered cells were used for immunoblotting assays, and the remainder were stained with 4′,6-diamidino-2-phenylindole to determine the percentages of GFP-positive cells with apoptotic nuclei (25).

GM701 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf serum. Cells were transfected with pRC/CMV-hBcl2, pcDNA3-Bcl-2Δa5a6 or pcDNA3-Bcl-2-Bax(Δa5a6) by a calcium-phosphate precipitation method and selected in 1.4 mg/ml (active) G418. Pools of stable transfectants were passaged and then cultured in 96-well plates for 12 h at a density of 1 × 10⁶ cells/0.1 ml/well. Fresh medium was exchanged, and 1 μm staurosporine (STS) was added to induce apoptosis. After 24 h, cell viability was determined by trypan blue dye exclusion assay.

**Yeast Cytotoxicity Assays**—EY48 strain cells were transformed by the lithium acetate method, using 1 μg of plasmid DNA (25, 26). Cells were then plated on histidine-deficient glucose-based minimal medium supplemented with other essential amino acids. Colonies were counted after culturing at 30 °C for 3 days. For the examination of Bcl-2-mediated rescue of yeast from Bax-induced cell death, EY48 cells were co-transformed with 1 μg of pGilda-Bax and 1 μg of pJG4–5-Bcl-2, pJG4–5-Bcl-2Δa5a6, pJG4–5-Bcl-2-Bax(Δa5a6), or pJG4–5-Bax-Bcl-2Δa5a6, and plated on both histidine- and tryptophan-deficient glucose-based medium to select for the plasmids. Single colonies of transformed yeast cells were re-streaked on galactose-containing medium to induce the GAL-1 promoters in these plasmids and cultured for 4 days (25).

**Yeast Two-hybrid Assays**—Protein-protein interactions were evaluated by yeast two-hybrid assay as described previously, using EY48 cells either for LEU2 or lacZ reporter gene assays, in conjunction with pEG202 (LexA DNA-binding domain) and pJG4–5 (B42 transactivation domain) plasmids (15, 17, 27). Growth on leucine-deficient medium was scored 4 days after spotting on minimal medium plates containing 2% galactose and 1% raffinose to induce expression of the transactivation.
domain-containing proteins from the GAL1 promoter in pHG4-5. Filter assays were similarly performed for β-galactosidase measurements, using cells plated on either galactose- or glucose-containing minimal medium supplemented with leucine. Blue color development was scored at 2 h after adding 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

**Immunoprecipitation and Immunoblotting Assays—** For co-immunoprecipitation experiments, 293T cells (2 × 10^6) were cultured for 12 h in 10 ml of medium. Fresh medium was exchanged, and 4 h later the cells were co-transfected with 10 μg of pRC/CMV-Bcl-2 and 10 μg of pcDNA3-HA-Bax, pcDNA3-HA-BaxΔΔ56, or pcDNA3-HA-Bax-Bcl-2Δ6, or with 10 μg of pcDNA3-Bax and 10 μg of pRC-CMV-Bcl-2, pcDNA3-Bcl-ΔΔ56, or pcDNA3-Bcl-2-BaxΔ56, by a calcium phosphate precipitation method. Four hours after transfections, fresh medium was exchanged and the cells were cultured for another 4 h before lysing in 0.6 ml of Nonidet P-40 lysis buffer (10 mM Hepes [pH 7.5] 142.5 mM KCl, 5 mM MgCl_2, 1 mM EDTA, 0.2% Nonidet P-40), containing 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After preclearing with 50 μl of Protein G-Sepharose at 4 °C for 1 h, immunoprecipitations were performed by incubating 0.2 ml of lysate with 20 μl of Protein G-Sepharose preabsorbed with 5 μg of anti-Bcl-2 mouse monoclonal antibody ascites (clone 4D7) or 10 μl of anti-Bax rabbit serum at 4 °C for 2 h (28, 29). After extensive washing in Nonidet P-40 lysis buffer, beads were boiled in 60 μl of Laemmli buffer. 20 μl of the eluted proteins were subjected to SDS-PAGE (12%) immunoblot analysis using anti-HA mouse monoclonal antibody conjugated with horseradish peroxidase (Boehringer Mannheim) or 4D7 anti-Bcl-2 mouse monoclonal antibody. For detection of Bcl-2, horseradish peroxidase-conjugated anti-mouse (Bio-Rad) antibody was employed. Immunodetection was achieved by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech) with exposure to x-ray film.

For immunoblot assays, whole cell lysates were normalized for total protein content, and immunoblot assays were performed as described previously using 0.1% (v/v) anti-LexA rabbit serum or either anti-Bax or anti-Bcl-2 rabbit serum (28, 29). After extensive washing with 0.1% (v/v) anti-LexA rabbit serum or either anti-Bax or anti-Bcl-2 rabbit serum (21, 29).

**Ion Channel Assays—** For immunoblot assays, whole cell lysates were normalized for total protein content, and immunoblot assays were performed as described previously using 0.1% (v/v) anti-LexA rabbit serum or either anti-Bax or anti-Bcl-2 rabbit serum (21, 29).

**RESULTS**

To examine the biological significance of the putative pore-forming α5 and α6 helices within Bcl-2 and Bax, mutants having α5 and α6 deleted were prepared. Alternatively, the α5 and α6 helices were swapped, thus generating chimeric proteins in which the α5 and α6 helices of Bax were replaced with those from Bcl-2 and vice versa (Fig. 1).

Previously, we demonstrated that deletion of the α5-α6 region from Bcl-2 abrogates the ability of the recombinant protein to form pH-dependent channels in liposomes in vitro (12). To explore the relevance of the α5-α6 region of Bax to its in vitro channel activity, recombinant Bax and BaxΔΔ56 proteins were produced in bacteria (without their C-terminal hydrophobic domains (DTM) for solubility purposes) and purified (data not shown). When applied at ~150 ng/ml to KCl-loaded unilamellar liposomes composed of 40:60 (mol/mol) DOPG:DOPC at pH 4.0 and ion efflux was monitored using a Cl– electrode as described previously (12). Triton X-100 (0.1%) was added to release residual KCl at the point indicated by arrow.

**Studies of Bcl-2 and Bax Mutants in Mammalian Cells—** When expressed in the human kidney epithelial cell line 293T by transient transfection, the wild-type Bax protein induced apoptosis in nearly half of the successfully transfected cells, as determined by 4',6-diamidino-2-phenylindole staining of GFP-expressing cells (Fig. 3). Similarly, apoptosis was also induced by transfection with plasmids encoding either the BaxΔ56 or Bax-Cleave-6 proteins into 293T cells. The BaxΔ56 and Bax-Cleave-6 proteins consistently induced a lower percentage of the transiently transfected 293T cells to undergo apoptosis when compared with wild-type Bax in experiments where varying amounts of these plasmid DNAs were employed (1, 2, 4, and 5 μg). However, immunoblot analysis of lysates prepared from the transfected 293T cells suggested that these mutant proteins may be produced at somewhat lower levels than the wild-type Bax protein (Fig. 3C; data not shown). These results indicate that the α5 and α6 helices of Bax are not absolutely required for apoptosis induction in 293T cells. Furthermore, introduction of the α5 and α6 helices from Bcl-2 into the Bax protein is insufficient to convert Bax from a killer to a protector protein.

The bioactivities of Bcl-2 mutant proteins lacking either α5 and α6 (Bcl-2Δα56) or which contained the corresponding α5-α6 region from Bcl-2 (Bax-Cleaveα56) were compared against the wild-type Bcl-2 protein in transient co-transfection assays to determine whether these proteins could suppress apoptosis induced by Bax. In contrast to wild-type Bcl-2, transfections performed with plasmids encoding the Bcl-2Δα56 or Bcl-2-Baxα56 proteins failed to suppress Bax-induced apoptosis in 293T cells (Fig. 3B). Immunoblot analysis of lysates prepared from these transiently transfected cells revealed at least comparable levels of production of the Bcl-2Δα56 and Bcl-2-Baxα56 proteins compared with wild-type Bcl-2 (Fig. 3D). Thus, removal of the α5-α6 region from Bcl-2 or replace-
All samples were normalized for total protein content (20 μg of pEGFP and 1 μg each of plasmids encoding the indicated proteins). Cells were collected 24 h after transfection, and the percentage of GFP positive cells with apoptotic nuclei was determined by 4',6-diamidino-2-phenylindole staining (mean ± S.D.; n = 3). Control, pcDNA3. B, 293T cells were transiently transfected with 0.5 μg of pEGFP and 2 μg total of the plasmids described below. Control, pcDNA3 (2 μg); Bax, pcDNA3-Bax (1 μg) and pcDNA3 (1 μg); Bax + Bcl-2, pcDNA3-Bax (1 μg) and pRC/CMV-Bcl-2 (1 μg); Bax + Bcl-2ΔΔα6 (1 μg); Bcl-2ΔΔα6, pcDNA3-Bax (1 μg) and pPCD/CMV-Bcl-2ΔΔα6 (1 μg); Bcl-2ΔΔα6, Bax + Bcl-2ΔΔα6, pcDNA3-Bax (1 μg) + pcDNA3-Bax-Bcl-2ΔΔα6 (1 μg). The percentage of apoptotic GFP-expressing cells is indicated (mean ± S.D.; n = 3). C and D, immunoblot analysis of lysates prepared from 293T cells transfected as in A and B. All samples were normalized for total protein content (20 μg/lane). The blot in C (lanes 1–4) was probed with anti-Bax antiserum, whereas anti-Bcl-2 antisera was employed for the blot in D (lanes 5–8). Blots were also incubated with anti-tubulin antibody to confirm loading of equivalent amounts of intact protein (lower panel). N.S. indicates nonspecific band.

The behavior of the Bcl-2 and Bax mutants was therefore tested in budding yeast. As in our prior reports (15, 17, 21), the failure of BaxΔΔα6 and Bax-Bcl-2ΔΔα6 to kill yeast was not attributable to poor expression of these proteins, as revealed by immunoblot assays performed using cells that had been cotransformed with Bcl-2 to nullify the cytotoxic actions of the wild-type Bax protein (Fig. 5B). Thus, the α5-α6 region of Bax is required for its cytotoxic activity in S. cerevisiae.

Although necessary for inducing yeast cell death, the α5-α6 helices of Bax are insufficient for mediating the lethal effects of Bax because the chimeric Bcl-2-Bax(α5α6) protein, in which the α5-α6 of Bax had been substituted for the corresponding region from Bax abolishes the cell death-inducing activity of Bax in yeast, as determined by a colony-forming assay, which measures relative numbers of viable clonogenic cells (15, 17, 21). As shown in Fig. 5A, yeast transformed with the plasmid encoding wild-type Bax formed very few colonies due to the lethal effect of Bax expression, whereas numerous colonies (typically >1000/μg of plasmid DNA) were formed when yeast were transformed with plasmids encoding BaxΔΔα6 or Bax-Bcl-2ΔΔα6. The failure of BaxΔΔα6 and Bax-Bcl-2ΔΔα6 to kill yeast was not attributable to poor expression of these proteins, as revealed by immunoblot assays performed using cells that had been cotransformed with Bcl-2 to nullify the cytotoxic actions of the wild-type Bax protein (Fig. 5B). Thus, the α5-α6 region of Bax is required for its cytotoxic activity in S. cerevisiae.

Fig. 3. Function and expression of Bax and Bcl-2 mutant proteins in 293T cells. A, 293T cells were transiently transfected with 0.5 μg of pEGFP and 1 μg each of plasmids encoding the indicated proteins. Cells were collected 24 h after transfection, and the percentage of GFP positive cells with apoptotic nuclei was determined by 4',6-diamidino-2-phenylindole staining (mean ± S.D.; n = 3). Control, pcDNA3. B, 293T cells were transiently transfected with 0.5 μg of pEGFP and 2 μg total of the plasmids described below. Control, pcDNA3 (2 μg); Bax, pcDNA3-Bax (1 μg) and pcDNA3 (1 μg); Bax + Bcl-2, pcDNA3-Bax (1 μg) and pRC/CMV-Bcl-2 (1 μg); Bax + Bcl-2ΔΔα6, pcDNA3-Bax (1 μg) and pPCD/CMV-Bcl-2ΔΔα6 (1 μg); Bcl-2ΔΔα6, pcDNA3-Bax (1 μg) + pcDNA3-Bax-Bcl-2ΔΔα6 (1 μg). The percentage of apoptotic GFP-expressing cells is indicated (mean ± S.D.; n = 3). C and D, immunoblot analysis of lysates prepared from 293T cells transfected as in A and B. All samples were normalized for total protein content (20 μg/lane). The blot in C (lanes 1–4) was probed with anti-Bax antiserum, whereas anti-Bcl-2 antisera was employed for the blot in D (lanes 5–8). Blots were also incubated with anti-tubulin antibody to confirm loading of equivalent amounts of intact protein (lower panel). N.S. indicates nonspecific band.

Fig. 4. Functional analysis of Bcl-2 mutants in GM701 cells. A, stable transfecants of GM701 cells were prepared by selection in G418 after transfection with the following plasmids: pcDNA3 (Vector), pRC-CMV-Bcl-2, pcDNA3-Bcl-2ΔΔα6, and pcDNA3-Bcl-2-Bax(α5α6). Cells were cultured at 1 × 10⁶ cells/0.1 ml/well in a 96-well dish and treated with or without STS (1 μM) for 24 h before assessing viability by trypan blue dye exclusion. B, immunoblot analysis of lysates prepared from stably transfected GM701 cells was performed, after normalization for total protein content (20 μg/lane). Blots were incubated with either anti-Bcl-2 antiserum (top panel) or anti-tubulin antibody (lower panel). N.S. indicates a nonspecific band.
region within the Bcl-2 protein, failed to display a lethal phenotype in yeast (Fig. 5A). Immunoblot analysis again confirmed production of this protein at levels equivalent to or greater than wild-type Bax (Fig. 5B), discounting poor expression as an explanation of the findings. Taken together, these observations indicate that the putative pore-forming α5 and α6 helices of Bax are necessary but insufficient for conferring a lethal phenotype in _S. cerevisiae_.

Bcl-2 can rescue yeast from the lethal effects of the wild-type Bax protein (15, 17, 21). To explore the role of the α5-α6 region of Bcl-2 for abrogation of Bax-induced cell death in yeast, cDNAs encoding wild-type or mutant Bcl-2 proteins were subcloned into a plasmid pG4–5 in which expression is driven from a conditional GAL1 promoter. These galactose-inducible plasmids were then co-transformed into yeast with pGilda-Bax, which also expresses wild-type Bax by GAL1 promoter, and the cells were plated initially on glucose to repress the GAL1 promoter. The resulting transformants were then streaked onto glucose (control) or galactose (test) plates. As shown in Fig. 5C, wild-type Bcl-2 effectively rescued yeast from the lethal effects of Bax, allowing growth of cells on galactose plates, whereas the Bcl-2Δα5α6 mutant lacking the putative pore-forming α5 and α6 helices and the Bcl-2-Bax(α5α6) chimera containing the α5-α6 region from Bax failed to nullify Bax-induced yeast cell death. Immunoblot analysis confirmed expression of these Bcl-2 mutant proteins at levels comparable to the normal Bcl-2 protein (data not shown). Thus, the α5-α6 region of Bcl-2 appears to be necessary for rescuing yeast from the cytotoxic actions of Bax. However, the putative pore-forming α-helices of Bcl-2 are evidently insufficient for rescue, as expression of the Bax-Bcl-2(α5α6) in which the α5-α6 region of Bcl-2 was inserted in place of the corresponding segment of Bax also failed to protect yeast from Bax-induced cell death (Fig. 5C). Thus, similar to the results obtained in mammalian cells, the α5-α6 region of Bcl-2 appears to be necessary but insufficient for the cytoprotective effect of Bcl-2.

### Analysis of Dimerization Capabilities of Bcl-2 and Bax Mutants—Bcl-2 and Bax are known to both homodimerize with themselves and heterodimerize with each other (1–3). We explored the effects of deleting the α5-α6 regions of Bcl-2 and Bax or swapping them on homo- and heterodimerization, using a yeast two-hybrid approach (Table I). For these assays, mutant and wild-type Bcl-2 and Bax proteins were expressed with appended N-terminal LexA DNA binding or B42 transactivation domains, but without their C-terminal membrane anchoring regions which could interfere with nuclear import. Removal of the membrane anchoring domain from the C terminus of Bax also abolishes its insertion into mitochondrial membranes, abolishing entirely or greatly reducing its cytotoxicity in yeast (11–13).

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"LexA-BaxΔα5α6 showed relatively high background and the signal of LexA-BaxΔα5α6 and B42-BaxΔα5α6 was similar to the negative control of LexA-BaxΔα5α6 and B42-Ras, thus resulting in a "a" score.

Thus, the α5-α6 region of Bcl-2 appears to be necessary for rescuing yeast from the cytotoxic actions of Bax. However, the putative pore-forming α-helices of Bcl-2 are evidently insufficient for rescue, as expression of the Bax-Bcl-2(α5α6) in which the α5-α6 region of Bcl-2 was inserted in place of the corresponding segment of Bax also failed to protect yeast from Bax-induced cell death (Fig. 5C). Thus, similar to the results obtained in mammalian cells, the α5-α6 region of Bcl-2 appears to be necessary but insufficient for the cytoprotective effect of Bcl-2.

### Table I

Summary of two-hybrid assay results

Binding results were deduced from yeast two-hybrid assays performed in both directions. Each cDNA was subcloned into pEG202 (for LexA-fusion) and pJG4–5 (for B42-fusion) vectors. Interactions were designated as "+" only if both directions showed positive binding signals (both β-galactose activity and Leu (−) assay) in comparison with negative control based on LexA-Fas and B42-Ras. The BaxΔα5α6, Bax-Bcl-2(α5α6), and wild-type Bax proteins retained the ability to interact with both Bcl-2 and Bax in yeast two-hybrid assays, consistent with reports indicating the ability of the second α-helix (BH3 domain) within this protein to bind to pockets found on the surface of other Bcl-2 family proteins (32). Thus, the α5-α6 region of Bax is not required for dimerization with the wild-type Bcl-2 or Bax proteins. The Bax-Bcl-2(α5α6) chimeric protein also retained the ability to interact with itself (Table I), implying that its lack of cell death inducing activity in yeast cannot be attributed to defective homodimerization. In contrast, the BaxΔα5α6 protein failed to interact with itself, consistent with structural studies that have implicated portions of the α5 and α6 helices in forming the base of the pocket into which the BH3 domain inserts (32).

Analysis of the α5-α6 region mutants of Bcl-2 revealed that all retained the ability to interact with Bcl-2 in yeast two-hybrid assays, implying that they were not grossly misfolded despite their apparent lack of bioactivity in both yeast and mammalian cells.

**Fig. 5. Functional analysis of Bax and Bcl-2 mutants in yeast.**
A, EGY48 strain yeast were transformed with 1 μg of either pEG202 parental DNA (Vector) or pEG202 plasmids containing cDNAs encoding the indicated proteins. Cells were plated on plasmid-selective medium (histidine-deficient). The number of independent colonies was counted after 3 days (mean ± S.D.; n = 3). B, lysates were prepared from EGY48 cells transformed as in A, normalized for total protein content (20 μg), and then subjected to SDS-PAGE (7.5%) immunoblot assay using anti-LexA antiserum for detection of either the LexA DNA fragment encoded in the pEG202 parental vector or LexA fusion proteins containing Bcl-2, Bax, or various Bax mutants as indicated. C, yeast cells were co-transformed with 1 μg of pGilda-Bax and 1 μg of pJG4–5 plasmids encoding wild-type Bcl-2, Bcl-2Δα5α6, Bcl-2-Bax(α5α6), or Bax-Bcl-2(α5α6). These plasmids utilize the GAL1 promoter to drive gene expression, resulting in repression of expression when cells are grown on glucose plates and gene induction on galactose. Transformants were first allowed to grow on histidine-deficient glucose plates (left) and then restreaked on galactose plates (right). Photographs represent growth obtained after culture at 30 °C for 4 days.
mammalian cells. However, neither Bcl-2Δ5-6 nor Bcl-2-
Bax(Δ5-6) homodimerized. The Bcl-2Δ5-6 deletion mutant also entirely failed to interact with Bax, and the Bcl-2-
Bax(Δ5-6) chimera displayed reduced interaction with Bax in
two-hybrid assays compared with the wild-type Bcl-2 protein (Table I).

To further explore the dimerization capabilities of the Δ5-6 region mutants of Bcl-2 and Bax, co-immunoprecipitation ex-
periments were performed using lysates from 293T cells that had been transiently transfected with plasmids encoding these proteins (Fig. 6). Consistent with the results of yeast two-
hybrid experiments, HA-epitope tagged versions of the wild-
type and hybrid experiments, HA-epitope tagged versions of the wild-

proteins as a likely explanation for the reduced ability of Bcl-
2-Bax(Δ5-6) to co-immunoprecipitate with Bax (Fig. 6). A va-
riety of control co-immunoprecipitations using HA-tagged or
untagged irrelevant proteins were performed, confirming the
specificity of the results presented in Fig. 6 (data not shown).

**DISCUSSION**

Bcl-2 and Bax are known to form ion channels in synthetic
membranes in vitro, and it has been speculated that the re-
gions predicted to coincide with the Δ5 and Δ6 helices of the homologous protein Bcl-X<sub>L</sub> are directly involved in this process (5). Here, we report the results of experiments in which the predicted Δ5 and Δ6 region of Bcl-2 and Bax were either deleted or swapped with each other. Our data provide evidence that: (a) Δ5 and Δ6 of Bcl-2 are required for its cytotoxic activity in both mammalian cells and yeast, (b) Δ5 and Δ6 of Bax are necessary for its cytoadective activity in yeast but not in mammalian cells, and (c) swapping the Δ5-Δ6 regions of Bcl-2 and Bax is insufficient for converting the phenotype of Bcl-2 to a killer and Bax to a protector. These results imply that, although necessary, these α-helices are apparently insufficient to explain why Bcl-2 is anti-apoptotic and Bax is pro-apoptotic in most cellular contexts.

The observation that the Δ5-Δ6 region is not required for
Bax-induced apoptosis in mammalian cells can presumably be explained by the ability of its BH3 domain (predicted second α-helix) to bind to and antagonize anti-apoptotic Bcl-2 family proteins (27). As shown here, the Δ5-Δ6 mutants of Bax retained the ability to co-immunoprecipitate with Bcl-2 and to interact with Bcl-2 in yeast two-hybrid assays. Previous studies have shown that overexpressing fragments of Bax or Bak that retain little more than their BH3 domain are sufficient to bind
Bcl-2 or Bcl-X<sub>L</sub> and to induce apoptosis in mammalian cells (33). Similarly, a Bcl-2 family subgroup comprising pro-apop-
totic proteins such as Bik, Bid, Bim, and Hrk has sequence similarity with other family members that is limited to the BH3 domain. Predicted structures for these proteins cannot be modeled on the Bcl-X<sub>L</sub> coordinates, implying that do not share structural similarity with the ion channel-forming proteins such as Bcl-2, Bcl-X<sub>L</sub>, and Bak (11–13). This BH3-mediated cell death mechanism may be relevant only in cells that express anti-apoptotic members of the Bcl-2 family, accounting for why Δ5-Δ6 region mutants of Bax were inactive in yeast that lack an identifiable Bcl-2 family protein. However, the observation that deletion of the Δ5-Δ6 region of Bax abrogates its cytotoxic function in yeast raises the possibility that Bax has two mecha-

isms for inducing apoptosis in mammalian cells: one that relies on BH3-mediated antagonism of proteins such as Bcl-2 and Bcl-X<sub>L</sub>, and another that maps to the Δ5 and Δ6 helices required for its channel-forming activity. Support for a second, BH3-independent mechanism of cell killing has been obtained through experiments involving BH3 domain mutants of Bax that failed to dimerize with Bcl-2 or Bcl-X<sub>L</sub>, and yet retained their pro-apoptotic function in mammalian cells (22, 34). A major question now is which of these two mechanisms for promoting apoptosis is quantitatively more important under physiological conditions where Bax is not artificially overexpressed.

In contrast to Bax, deletion or substitution of the putative channel forming Δ5 and Δ6 helices of Bcl-2 abolished its cyto-

protective function in both mammalian cells and yeast, indic-
atating that this region is indispensable for function of the Bcl-2 protein. Previously, we reported that deletion of Δ5 and Δ6 from Bcl-2 abrogates its ability to form ion channels in liposomes and planar bilayers in vitro (12). Thus, it is possible that channel activity is required for Bcl-2 to promote cell survival and diminish Bax-induced cell death. Unfortunately, multiple at-
Cytoprotection by Bcl-2 Requires the Pore-formation Domain

Cytoprotective activity of Bcl-2 was demonstrated to be insufficient for promoting cell survival, as replacement of the Bcl-2 region of Bax for the corresponding region of Bax caused a decrease in the kinetic activity. Moreover, the Bcl-2-Bax chimera observed in yeast is involved in such protein interactions. In this regard, Bcl-2 has been reported to bind directly or at least to some extent to dimerization among Bcl-2 family proteins or interactions with other types of non-homologous proteins such as those involved in caspase regulation.

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REFERENCES


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