UC Office of the President
Recent Work

Title

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

Permalink

Authors

Matsuyama, Shigemi
Schende, Sharon L.
Xie, Zhihua
et al.

Publication Date

DOI

Peer reviewed

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

Shigemi Matsuyama, Sharon L. Schendel, Zhihua Xie, and John C. Reed‡

From the Burnham Institute, Program on Apoptosis & Cell Death Research, La Jolla, California 92037

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-2Δα5α6 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. BaxΔα5α6 and Bax-Bcl-2(α5α6) 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-X$_L$ 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-X$_L$, 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-X$_L$ 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-X$_L$ 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-X$_L$, 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 cytoprotective (Bcl-2, Bcl-X$_L$) and cytotoxic (Bax) members of the Bcl-2 family differ. In general, Bcl-2 and Bcl-X$_L$ tend to form channels having low conductance, display 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-X$_L$ 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-X$_L$ 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-X$_L$, 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-X$_L$ 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,

‡ To whom correspondence should be addressed: Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-646-3140; Fax: 619-646-3194; E-mail: jreed@burnham-inst.org.

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 $\alpha 5$ and $\alpha 6$ helices are devoid of cytotoxic activity in yeast. Bcl-2 and Bcl-X_L 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 $\alpha 5$ and $\alpha 6$ 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 $\alpha 5$ and $\alpha 6$ helices of Bcl-2 and Bax are necessary but insufficient for determining the opposing phenotypes of these proteins.

<center>MATERIALS AND METHODS</center>

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 *Eco*RI (5′ end) and *Xho*I (3′ end) sites in pEG202, pJG4–5, pcDNA3, or pcDNA3-HA plasmids. The following mutagenic primers were used in combination with the wild-type Bcl-2 forward (for pEG202, pJG4–5: 5′-GCGGAATTCA-TGGCGCACGCTGGGAGAACA-3′; for pcDNA3: 5′-GCGGAATTCGCC-ACCATGGCGCACGCTGGGAGAACA-3′) and reverse (with C-terminal transmembrane domain (TM)[1]: 5′-ATTCTCGAGTCACTTGTGGCCCA-GATAGGC-3′; without TM: 5′-CGCCTCGAGTCAAGTCTTCAGAGAC-AGCCAGGA-3′), for wild-type Bax forward (for pEG202, pJG4–5, or pcDNA3-HA: 5′-GCGGAATTCATGGACGGGTCCGGGGAGGAG-3′; for pcDNA3: 5′-GCGGAATTCGCCACCATGGACGGGTCCGGGGAGGAG-3′) and reverse (with TM: 5′-ATTCTCGAGTCAGCCCATCTTCTTCCA-GAT-3′; without TM: 5′-ATTCTCGAGTCAGGGCGTCCCAAAGTAGG-AGAG-3′), for Bcl-2Δα5α6, 5′-CTGCACACCTGGATCCAGGATAACG-GA-3′ (forward) and 5′-CCAGGTGTGCAGCACCCCGTGCCTGAAGA-GCTC-3′ (reverse), and for Bax2Δα5α6, 5′-GACGGCAACTTCGACCA-GGGTGGTTGGGACGGC-3′ (forward) and 5′-GAAGTTGCCGTCAGA-AAACATGTCAGC-3′ (reverse). For the construction of Bcl-2-Bax or Bax-Bcl-2 chimeras, first a *Sac*I site was introduced into the Bax cDNA by two-step polymerase chain reaction using 5′-GCAGCTGAGCTCTT-TTCTGACGGCAACTTCAAC-3′ (forward) and 5′-AGAAAAGAGCTCA-GCTGCCACTCGGAAAAAGAC-3′ (reverse) with the above primers for wild-type Bax. Then, the region of the Bcl-2 cDNA and Bax cDNAs between the *Sac*I and *Bam*HI sites was swapped. For the production of recombinant GST-Bax(ΔTM) and GST-BaxΔα5α6(ΔTM), cDNAs encoding Bax(ΔTM) and BaxΔα5α6(ΔTM) were subcloned between *Eco*RI (5′ end) and *Xho*I (3′ end) sites in pGEX-4T-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 immunoblot
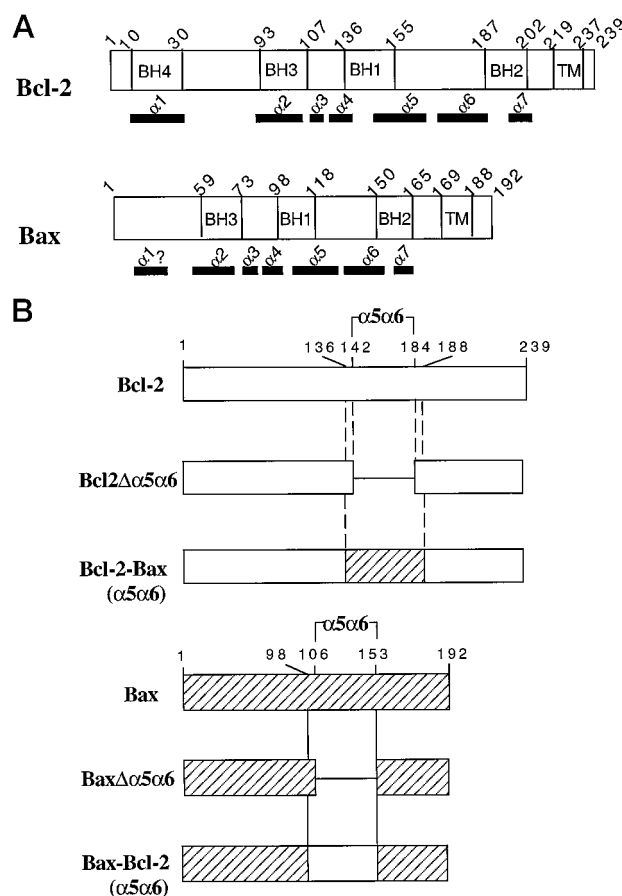
FIG. 1. **Bcl-2 and Bax mutants and chimeras.** The predicted positions of the α-helical regions within the human Bcl-2 and Bax proteins are depicted (*A*), and the $\alpha 5$-$\alpha 6$ region deletion mutants and chimeras of Bcl-2 and Bax are illustrated (*B*). The boundaries of the regions corresponding to the $\alpha 5$ and $\alpha 6$ helices were deduced from Ref. 4 and confirmed independently by modeling the human Bcl-2 and Bax proteins on the Bcl-X_L coordinates (5). *Numbers* indicate amino acid positions. The *open bars* refer to Bcl-2 and the *hatched bars* to Bax.

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-2Δα5α6, or pcDNA3-Bcl-2-Bax(α5α6) by a calcium-phosphate precipitation method and selected in 1.4 mg/ml (active) G418. Pools of stable transfectants were passaged and then were cultured in 96-well plates for 12 h at a density of 1×10^4 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—EGY48 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, EGY48 cells were co-transformed with 1 μg of pGilda-Bax and 1 μg of pJG4–5-Bcl-2, pJG4–5-Bcl-2Δα5α6, pJG4–5-Bcl-2-Bax(α5α6), or pJG4–5-Bax-Bcl-2(α5α6), 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 EGY48 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

---

[1] The abbreviations used are: TM, transmembrane; DOPC, 1,2-dioleoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; STS, staurosporine.

domain-containing proteins from the GAL1 promoter in pJG4–5. 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-BaxΔα5α6, or pcDNA3-HA-Bax-Bcl-2(α5α6), or with 10 μg of pcDNA3-Bax and 10 μg of pRc-CMV-Bcl-2, pcDNA3-Bcl-2Δα5α6, or pcDNA3-Bcl-2-Bax(α5α6), 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 MgCl₂, 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, immnoprecipitations 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 and 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 (21, 29).

Ion Channel Assays—Recombinant GST-Bax (ΔTM) and GST-BaxΔα5α6 (ΔTM) proteins were produced from pGEX-4T-1 in *E. coli* (BL21 (DE3) strain) bacteria and purified by glutathione-Sepharose affinity chromatography essentially as described (12, 30, 31). GST was removed by cleavage with thrombin, and the Bax(ΔTM) and BaxΔα5α6 (ΔTM) proteins were subsequently purified by ion-exchange chromatography (12, 30, 31) and dialyzed into 20 mM Tris-HCl, pH 8.0. Folding of the purified proteins was confirmed by circular dichroism measurements carried out on an AVIV 60DS spectropolarimeter. Proteins were assayed for channel activity on KCl-loaded unilammelar liposomes composed of 60% DOPC (1,2-dioleoylphosphatidylcholine) and 40% DOPG (1,2-dioleoylphosphatidylglycerol) at pH 4.0, measuring Cl⁻ ion efflux as described (12).

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 abolishes 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 BaxΔα5α6 proteins were produced in bacteria (without their C-terminal hydrophobic domains (ΔTM) for solubility purposes) and purified (data not shown). When applied at ~150 ng/ml to KCl-loaded unilammelar liposomes under conditions previously shown to be permissive for channel formation by Bcl-2 family proteins (11–14), Bax (ΔTM) induced striking ion efflux (Fig. 2). In contrast, the BaxΔα5α6 (ΔTM) protein exhibited little or no channel activity under the same conditions. Additional experiments revealed that Bax channel formation was dependent on acidic pH (optimal pH ~4.0) and the presence of acidic lipids within liposomes (DOPG), consistent with prior studies of Bax and other Bcl-2 family proteins (11–14). In contrast, the BaxΔα5α6
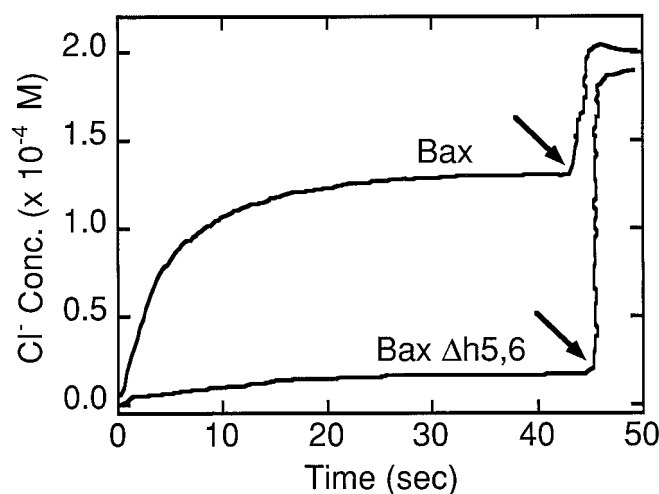

FIG. 2. **Bax channel activity requires α5-α6 region.** Bax (ΔTM) or BaxΔα5α6 (ΔTM) were added at 150 ng/ml to KCl-loaded unilammelar liposomes composed of 40:60 (mol/mol) DOPG:DOPC at pH 4.0 and ion efflux was monitored using a Cl⁻ electrode as described previously (12). Triton X-100 (0.1%) was added to release residual KCl at the point indicated by *arrow*.

(ΔTM) protein induced either negligible ion-efflux or (at higher concentrations) exhibited only nonspecific effects, producing similar amounts of Cl⁻ release at both neutral and acidic pH and regardless of whether lipsomes contained acidic lipids (DOPG) or were composed entirely of neutral lipids (DOPC) (data not shown). Although the absence of the C-terminal membrane anchoring domain may reduce the efficiency, these experiments nevertheless demonstrate α5-α6-dependent channel formation by Bax *in vitro*.

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 BaxΔα5α6 or Bax-Bcl-2(α5α6) proteins into 293T cells. The BaxΔα5α6 and Bax-Bcl-2(α5α6) 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 8 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. 3*C*; 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-2Δα5α6) or which contained the corresponding α5-α6 region from Bax (Bcl-2-Bax(α5α6)) 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-2Δα5α6 or Bcl-2-Bax(α5α6) proteins failed to suppress Bax-induced apoptosis in 293T cells (Fig. 3*B*). Immunoblot analysis of lysates prepared from these transiently transfected cells revealed at least comparable levels of production of the Bcl-2Δα5α6 and Bcl-2-Bax(α5α6) proteins compared with wild-type Bcl-2 (Fig. 3*D*). Thus, removal of the α5-α6 region from Bcl-2 or replace-
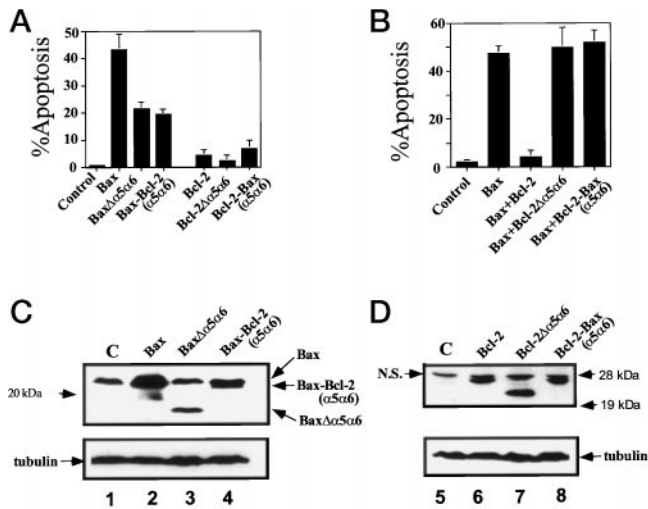
FIG. 3. **Function and expression of Bax and Bcl-2 mutant proteins in 293T cells.** *A*, 293T cells were transiently transfected with 0.5 μg of pEGFP and 1 μg each of plasmids encoding the indicated proteins. Cells were collected 24 h after transfection, and the percentage of GFP positive cells with 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-2Δα5α6*, pcDNA3-Bax (1 μg) and pcDNA3-Bcl-2Δα5α6 (1 μg); *Bax+Bcl-2-Bax(α5α6)*, pcDNA3-Bax (1 μg) + pcDNA3-Bax-Bcl-2(α5α6) (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 antiserum 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 non-specific band.

ment of the corresponding region from Bax abolishes the ability of Bcl-2 to block Bax-mediated apoptosis. When expressed in 293T cells without co-transfection of Bax, neither the Bcl-2Δα5α6 nor the Bcl-2-Bax(α5α6) protein induced significant apoptosis (Fig. 3*A*), arguing that substitution of the α5-α6 region of Bax does not convert Bcl-2 into a killer protein.

To further explore the function of the Bcl-2Δα5α6 and Bcl-2-Bax(α5α6) proteins, their ability to inhibit STS-induced apoptosis in GM701 cells was compared with the wild-type Bcl-2 protein. Treatment with this broad-specificity kinase inhibitor induced apoptosis in ~70% of GM701 cells (Fig. 4). Wild-type Bcl-2 potently suppressed STS-induced apoptosis. In contrast, neither Bcl-2Δα5α6 nor Bcl-2-Bax(α5α6) interfered with STS-induced apoptosis (Fig. 4*A*), despite expression of these mutant proteins at levels equivalent to or greater than the wild-type Bcl-2 protein (Fig. 4*B*). Expression of Bcl-2Δα5α6 or Bcl-2-Bax(α5α6) in GM701 cells did not induce apoptosis in the absence of STS, indicating that these Bcl-2 mutant proteins are not intrinsically cytotoxic (data not shown). Taken together, these observations indicate that the α5-α6 region of Bcl-2 is essential for its anti-apoptotic activity in mammalian cells.

Studies of Bcl-2 and Bax Mutant Proteins in Yeast—Ectopic expression of Bax in *S. cerevisiae* has been shown to induce cell death through a Bcl-2-suppressible mechanism (15, 17, 21). The behavior of the Bcl-2 and Bax mutants was therefore tested in budding yeast. As in our prior reports (15, 17, 21, 25), wild-type and mutant versions of Bcl-2 and Bax were expressed as LexA fusion proteins, using the LexA sequences as an epitope tag for monitoring expression levels.

In contrast to the results obtained in mammalian cells, deletion of the α5-α6 region from Bax or substitution of the
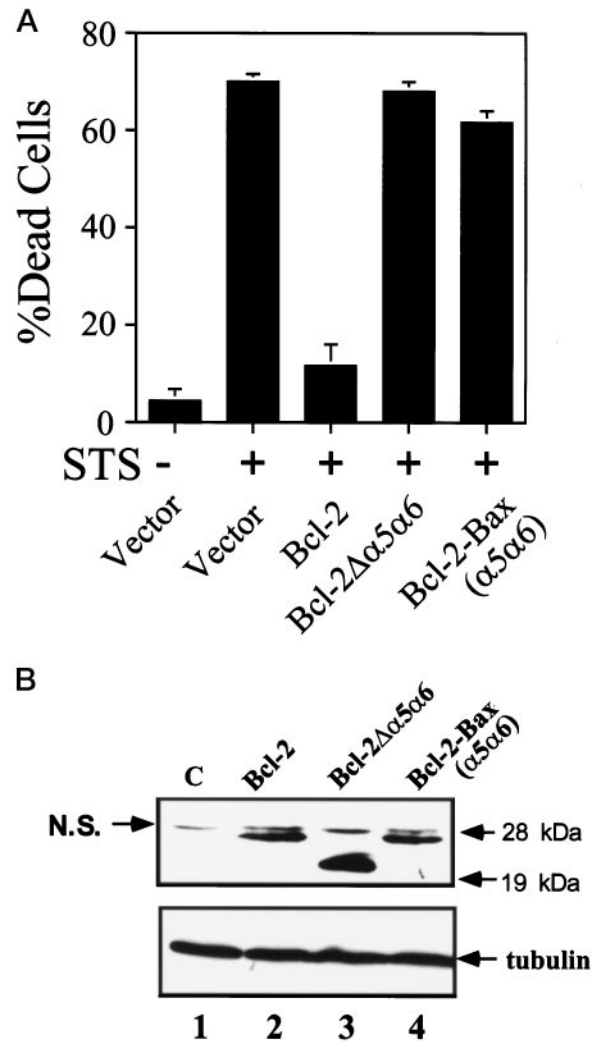

FIG. 4. **Functional analysis of Bcl-2 mutants in GM701 cells.** *A*, stable transfectants of GM701 cells were prepared by selection in G418 after transfection with the following plasmids: pcDNA3 (*Vector*), pRc-CMV-Bcl-2, pcDNA3-Bcl-2Δα5α6, and pcDNA3-Bcl-2-Bax(α5α6). Cells were cultured at 1 × 10⁴ 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.

corresponding region from Bcl-2 abolished the cell death-inducing activity of Bax in yeast, as determined by a colony-forming assay, which measures relative numbers of viable clonigenic cells (15, 17, 21). As shown in Fig. 5*A*, 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 BaxΔα5α6 or Bax-Bcl-2(α5α6). The failure of BaxΔα5α6 and Bax-Bcl-2(α5α6) 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. 5*B*). 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
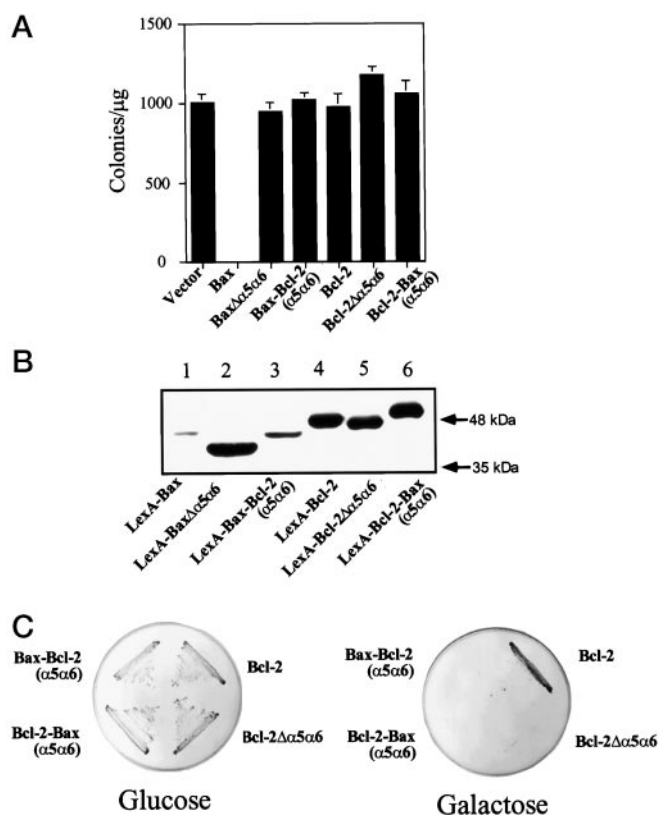
FIG. 5. **Functional analysis of Bax and Bcl-2 mutants in yeast.** *A*, EGY48 strain yeast were transformed with 1 μg of either pEG202 parental DNA (*Vector*) or pEG202 plasmids containing cDNAs encoding the indicated proteins. Cells were plated on plasmid-selective medium (histidine-deficient). The 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.

region within the Bcl-2 protein, failed to display a lethal phenotype in yeast (Fig. 5*A*). Immunoblot analysis again confirmed production of this protein at levels equivalent to or greater than wild-type Bax (Fig. 5*B*), 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 pJG4–5 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 either glucose (control) or galactose (test) plates. As shown in Fig. 5*C*, wild-type Bcl-2 effectively rescued yeast from the lethal effects of Bax, allowing growth of cells on galactose plates,

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.

	Bcl-2	Bcl-2Δα5α6	Bcl-2-Bax(α5α6)	Bax	BaxΔα5α6	Bax-Bcl-2(α5α6)
Bcl-2	+	+	+	+	+	+
Bcl-2Δα5α6	+	−	−	−	−	−
Bcl-2-Bax(α5α6)	+	−	−	±	+	+
Bax	+	−	±	+	+	+
BaxΔα5α6	+	−	+	+	−[a]	+
Bax-Bcl-2(α5α6)	+	−	+	+	+	+

[a] LexA-BaxΔα5α6 showed relatively high background and the signal of LexA-BaxΔα5α6 and B42-BaxΔα5α6 was similar to the negative control of LexA-Bax Δα5α6 and B42-Ras, thus resulting in a "−" score.

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. 5*C*). 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).

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
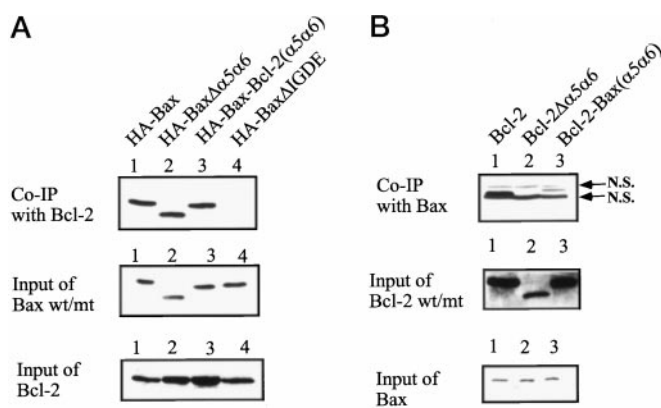
FIG. 6. **Analysis of dimerization of Bax and Bcl-2 mutant proteins by co-immunoprecipitation.** *A*, 293T cells were transfected with pRc-CMV-Bcl2 (*lanes 1–4*) and pcDNA3-HA-Bax (*lane 1*), pcDNA3-HA-BaxΔα5α6 (*lane 2*), pcDNA3-HA-Bax-Bcl-2(α5α6) (*lane 3*), or pcDNA3-HA-BaxΔIGDE (*lane 4*). Two days after transfection, cell lysates were prepared and subjected to immunoprecipitation using anti-Bcl-2 monoclonal antibody. Immune complexes were analyzed by SDS-PAGE (12%) immunoblot assay, using anti-HA antibody (*upper panel*) or anti-Bcl-2 antiserum for immunodetection (*bottom panel*). *Middle panel* represents lysates from the same cells that were normalized for total protein content (20 μg/lane) and analyzed for relative levels of Bax protein by immunoblotting using anti-Bax antiserum, confirming production of wild-type Bax and all Bax mutants in the transfected cells. *B*, 293T cells were transiently transfected with pcDNA3-Bax (*lanes 1–3*) and pRc/CMV-h Bcl-2 (*lane 1*), pcDNA3-Bcl-2Δα5α6 (*lane 2*), or pcDNA3-Bcl-2-Bax(α5α6) (*lane 3*). Immunoprecipitations were performed using anti-Bax antiserum, followed by SDS-PAGE (12%) immunoblot analysis using anti-Bcl-2 monoclonal antibody (*upper panel*) or anti-Bax antiserum (*bottom panel*) for detection. In the *middle panel*, lysates were normalized for total protein content (20 μg) and subjected to immunoblot assay using anti-Bcl-2 antiserum, confirming production of wild-type Bcl2 and all Bcl-2 mutant proteins. Negative controls to confirm the specificity of these protein interactions included immunoprecipitations performed with HA-BaxΔIGDE and anti-HA antibodies and blotting with anti-Bcl-2 antibody (data not shown).

mammalian cells. However, neither Bcl-2Δα5α6 nor Bcl-2-Bax(α5α6) homodimerized. The Bcl-2Δα5α6 deletion mutant also entirely failed to interact with Bax, and the Bcl-2-Bax(α5α6) 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 α5-α6 region mutants of Bcl-2 and Bax, co-immunoprecipitation experiments 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 α5-α6 region mutants of Bax retained the ability to co-immunoprecipitate with Bcl-2, and were recovered in anti-Bcl-2 immune complexes with approximately equivalent efficiency relative to each other (Fig. 6). As a control, experiments were also performed with a mutant of Bax in which a well conserved I-G-D-E amino acid sequence found within the BH3 domain had been deleted. Consistent with our previous studies of this mutant, no co-immunoprecipitation with Bcl-2 was detected, thus confirming the specificity of the results (24).

Analysis of the Bcl-2 mutants also reinforced the findings of yeast two-hybrid assays. When expressed in 293T cells, the Bcl-2Δα5α6 deletion mutant failed to co-immunoprecipitate with Bax and the Bcl-2-Bax(α5α6) chimeric protein displayed reduced co-immunoprecipitation relative to the wild-type Bcl-2 proteins. Immunoblot analysis of the same lysates indicated that the wild-type Bcl-2 and Bcl-2-Bax(α5α6) proteins were produced at comparable levels in transiently transfected 293T cells, excluding differences in the relative amounts of these

proteins as a likely explanation for the reduced ability of Bcl-2-Bax(α5α6) to co-immunoprecipitate with Bax (Fig. 6). A variety 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 regions predicted to coincide with the α5 and α6 helices of the homologous protein Bcl-X$_L$ are directly involved in this process (5). Here, we report the results of experiments in which the predicted α5 and α6 region of Bcl-2 and Bax were either deleted or swapped with each other. Our data provide evidence that: (*a*) α5 and α6 of Bcl-2 are required for its cytoprotective activity in both mammalian cells and yeast, (*b*) α5 and α6 of Bax are necessary for its cytodestructive activity in yeast but not in mammalian cells, and (*c*) swapping the α5-α6 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 α5-α6 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 α5-α6 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$_L$ and to induce apoptosis in mammalian cells (33). Similarly, a Bcl-2 family subgroup comprising pro-apoptotic 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$_L$ coordinates, implying that do not share structural similarity with the ion channel-forming proteins such as Bcl-2, Bcl-X$_L$, and Bax (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 α5-α6 region mutants of Bax were inactive in yeast that lack an identifiable Bcl-2 family protein. However, the observation that deletion of the α5-α6 region of Bax abrogates its cytotoxic function in yeast raises the possibility that Bax has two mechanisms for inducing apoptosis in mammalian cells: one that relies on BH3-mediated antagonism of proteins such as Bcl-2 and Bcl-X$_L$ and another that maps to the α5 and α6 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$_L$, 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 α5 and α6 helices of Bcl-2 abolished its cytoprotective function in both mammalian cells and yeast, indicating that this region is indispensable for function of the Bcl-2 protein. Previously, we reported that deletion of α5 and α6 from Bcl-2 abrogates its ability to form ion channels in lipsomes 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-

tempts to produce the Bcl-2-Bax($\alpha5\alpha6$) chimeric protein in bacteria for ion channel studies were unsuccessful due to protein instability and insolubility, thus precluding a comparison with the wild-type Bcl-2 protein *in vitro* channel activity.[2] Although we cannot exclude the possibility that substitution the $\alpha5$-$\alpha6$ region of Bcl-2 for the corresponding region of Bax caused a gross misfolding of the protein when expressed in mammalian cells or yeast, the Bcl-2-Bax($\alpha5\alpha6$) chimeric protein appeared to be stable, accumulating to levels comparable to the wild-type Bcl-2 protein. Bcl-2-Bax($\alpha5\alpha6$) also retained its ability to dimerize with Bax, albeit with reduced efficiency compared with wild-type Bcl-2. Moreover, the Bcl-2-Bax($\alpha5\alpha6$) chimera retained the ability to interact with Bcl-2 in yeast two-hybrid assays. Thus, dimerization with Bax or Bcl-2 appears to be insufficient for maintaining the cytoprotective function of the Bcl-2 protein in either mammalian cells or yeast. It will be of interest to identify other proteins with which this Bcl-2-Bax($\alpha5\alpha6$) chimera and the wild-type Bcl-2 protein interact. In this regard, Bcl-2 has been reported to bind directly or at least participate in protein complexes containing several types of non-homologous proteins in mammalian cells, including the kinase Raf-1 (35), the phosphatase calcineurin (36), the Hsp70/Hsc70-regulator BAG-1 (37), the caspase-binding protein Bap31 (38), the spinal muscular atrophy protein (SMN) (39), and others (1). Although the CED-4 homolog Apaf-1 (without WD domain) has recently received much attention for its ability to bind both caspases and Bcl-X_L (40, 41), we have been unable to detect interactions between Bcl-2 and Apaf-1 using numerous experimental approaches,[2] making it unlikely that differential binding of wild-type and chimeric Bcl-2 to Apaf-1 accounts for the ability of the former and failure of the latter to promote cell survival in mammalian cells. Moreover, as the completed genome of *S. cerevisiae* reveals no Apaf-1 homologs or caspases, it is highly unlikely that the cytoprotective function of Bcl-2 observed in yeast is involved in such protein interactions.

Although necessary for function of Bcl-2, the $\alpha5$-$\alpha6$ region appears to be insufficient for promoting cell survival, as replacing the $\alpha5$-$\alpha6$ region of Bax with this segment of Bcl-2 did not convert Bax to a cytoprotective protein. Likewise, although the $\alpha5$-$\alpha6$ region of Bax was necessary for its cytotoxic activity in yeast, engineering these predicted α-helices into the Bcl-2 protein was insufficient for switching its phenotype. Several previous reports have suggested that the BH3 domain (second α-helix) is an important determinant of the functions of pro-apoptotic Bcl-2 family proteins in mammalian cells and yeast (1, 24, 27). The BH4 domain (first α-helix in Bcl-X_L structure) of anti-apoptotic Bcl-2 family proteins has also been shown to be important for their cytoprotective function in both yeast and mammalian cells (1, 17, 35). Therefore, the opposing phenotypes of Bcl-2 and Bax presumably require both the $\alpha5$-$\alpha6$ region and additional domains such as BH3 or BH4. Determination of the topology of the Bcl-2 and Bax proteins when integrated into membranes in channel-forming conformation will help to reveal whether these other domains such as BH3 and BH4 directly contribute to channel formation by integrating perpendicularly through membranes as proposed for $\alpha5$ and $\alpha6$, *versus* regulating cell death through their contributions to dimerization among Bcl-2 family proteins or interac-

tions with other types of non-homologous proteins such as those involved in caspase regulation.

REFERENCES

1. Reed, J. C. (1997) *Nature* **387,** 773–776
2. Nuñuz, G., and Clarke, M. F. (1994) *Trends Cell Biol.* **4,** 399–403
3. Reed, J. C. (1994) *J. Cell Biol.* **124,** 1–6
4. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Changs, B. S., Thompson, C. B., Wong, S., Ng, S., and Fesik, S. W. (1996) *Nature* **381,** 335–341
5. Schendel, S., Montal, M., and Reed, J. C. (1998) *Cell Death Differ.* **5,** 372–380
6. Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. (1993) *Cancer Res.* **53,** 4701–4714
7. Lithgow, T., van Driel, R., Bertram, J. F., and Strasser, A. (1994) *Cell Growth Differ.* **3,** 411–417
8. Kagan, B. L., Finkelstein, A., and Colombini, M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4950–4954
9. Donovan, J. J., Simon, M. I., and Montal, M. (1985) *J. Biol. Chem.* **260,** 8817–8823
10. Cramer, W. A., Heymann, J. B., Schendel, S. L., Deriy, B. N., Cohen, F. S., Elkins, P. A., and Stauffacher, C. V. (1995) *Annu. Rev. Biophys. Biomol. Struct.* **24,** 611–641
11. Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997) *Nature* **385,** 353–357
12. Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M., and Reed, J. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5113–5118
13. Schlesinger, P., Gross, A., Yin, X.-M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11357–11362
14. Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.-J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R., and Martinou, J.-C. (1997) *Science* **277,** 370–372
15. Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H.-G., and Reed, J. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9238–9242
16. Bodrug, S. E., Aimé-Sempé, C., Sato, T., Krajewski, S., Hanada, M., and Reed, J. C. (1995) *Cell Death Differ.* **2,** 173–182
17. Hanada, M., Aimé-Sempé, C., Sato, T., and Reed, J. C. (1995) *J. Biol. Chem.* **270,** 11962–11968
18. Greenhalf, W., Stephan, C., and Chaudhuri, B. (1996) *FEBS Lett.* **380,** 169–175
19. Ink, B., Zornig, M., Baum, B., Hajibagheri, N., James, C., Chittenden, T., and Evan, G. (1997) *Mol. Cell. Biol.* **17,** 2468–2474
20. Jürgensmeier, J. M., Krajewski, S., Armstrong, R., Wilson, G. M., Oltersdorf, T., Fritz, L. C., Reed, J. C., and Ottilie, S. (1997) *Mol. Biol. Cell* **8,** 325–229
21. Zha, H., Fisk, H. A., Yaffe, M. P., Mahajan, N., Herman, B., and Reed, J. C. (1996) *Mol. Cell. Biol.* **16,** 6494–6508
22. Zha, H., and Reed, J. C. (1997) *J. Biol. Chem.* **272,** 31482–31488
23. Longo, V. D., Ellerby, L. M., Bredesen, D. E., Valentine, J. S., and Gralla, E. B. (1997) *J. Cell Biol.* **137,** 1581–1588
24. Zha, H., Fisk, H. A., Yaffe, M. P., and Reed, J. C. (1996) *Mol. Cell. Biol.* **16,** 6494–6508
25. Matsuyama, S., Xu, Q., Velours, J., and Reed, J. C. (1998) *Mol. Cell* **1,** 327–336
26. Schiestl, R. H., and Giest, R. D. (1989) *Curr. Genet.* **16,** 339–346
27. Zha, H., Aime-Sempe, C., Sato, T., and Reed, J. C. (1996) *J. Biol. Chem.* **271,** 7440–7444
28. Reed, J. C., Tanaka, S., Cuddy, M., Cho, D., Smith, J., Kallen, R., Saragovi, H. U., and Torigoe, T. (1992) *Anal. Biochem.* **205,** 70–76
29. Krajewski, S., Blomvqvist, C., Franssila, K., Krajewska, M., Wasenius, V.-M., Niskanen, E., and Reed, J. C. (1995) *Cancer Res.* **55,** 4471–4478
30. Marzo, I., Brenner, C., Zamzami, N., Susin, S., Beutner, G., Brdiczka, D., Xie, Z., Reed, J., and Kroemer, G. (1998) *J. Exp. Med.* **187,** 1261–1271
31. Zamzami, N., Marzo, I., Susin, S., Brenner, C., Larochette, N., Marchetti, P., Reed, J. C., Reinhard, K., and Kroemer, G. (1998) *Oncogene* **16,** 1055–1063
32. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) *Science* **275,** 983–986
33. Simonen, M., Keller, H., and Heim, J. (1997) *Eur. J. Biochem.* **249,** 85–91
34. Simonian, P. L., Grillot, D. A. M., Merino, R., and Nunez, G. (1996) *J. Biol. Chem.* **271,** 22764–22772
35. Wang, H.-G., Rapp, U. R., and Reed, J. C. (1996) *Cell* **87,** 629–638
36. Shibasaki, F., Kondo, E., Akagi, T., and McKeon, F. (1997) *Nature* **386,** 728–731
37. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) *Cell* **80,** 279–284
38. Ng, F. W. H., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. (1997) *J. Cell Biol.* **39,** 327–338
39. Iwahashi, H., Eguchi, Y., Yasuhara, N., Hanafusa, T., Matsuzawa, Y., and Tsujimoto, Y. (1997) *Nature* **390,** 413–417
40. Pan, G., O'Rourke, K., and Dixit, V. M. (1998) *J. Biol. Chem.* **273,** 5841–5845
41. Hu, Y., Benedict, M., Wu, D., Inohara, N., and Nunez, G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4386–4391

---

[2] S. Matsuyama, S. L. Schendel, Z. Xie, and J. C. Reed, unpublished observations.

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

Shigemi Matsuyama, Sharon L. Schendel, Zhihua Xie and John C. Reed

Access the most updated version of this article at http://www.jbc.org/content/273/47/30995

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 22 of which can be accessed free at
http://www.jbc.org/content/273/47/30995.full.html#ref-list-1