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Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system

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ABSTRACT **Interactions of the Bcl-2 protein with itself and other members of the Bcl-2 family, including Bcl-X-L, Bcl-X-S, Mcl-1, and Bax, were explored with a yeast two-hybrid system. Fusion proteins were created by linking Bcl-2 family proteins to a LexA DNA-binding domain or a B42 trans-activation domain. Protein–protein interactions were examined by expression of these fusion proteins in *Saccharomyces cerevisiae* having a *lacZ* (β-galactosidase) gene under control of a LexA-dependent operator. This approach gave evidence for Bcl-2 protein homodimerization. Bcl-2 also interacted with Bcl-X-L and Mcl-1 and with the dominant inhibitors Bax and Bcl-X-S. Bcl-X-L displayed the same pattern of combinatorial interactions with Bcl-2 family proteins as Bcl-2. Use of deletion mutants of Bcl-2 suggested that Bcl-2 homodimerization involves interactions between two distinct regions within the Bcl-2 protein, since a LexA protein containing Bcl-2 amino acids 83–218 mediated functional interactions with a B42 fusion protein containing Bcl-2 amino acids 1–81 but did not complement a B42 fusion protein containing Bcl-2 amino acids 83–218. In contrast to LexA/Bcl-2 fusion proteins, expression of a LexA/Bax protein was lethal to yeast. This cytotoxicity could be abrogated by B42 fusion proteins containing Bcl-2, Bcl-X-L, or Mcl-1 but not those containing Bcl-X-S (an alternatively spliced form of Bcl-X that lacks a well-conserved 63-amino acid region). The findings suggest a model whereby Bax and Bcl-X-S differentially regulate Bcl-2 function, and indicate that requirements for Bcl-2/Bax heterodimerization may be different from those for Bcl-2/Bcl-2 homodimerization.**

The *bcl-2* gene becomes dysregulated in a wide variety of human cancers and contributes to neoplastic cell expansion by prolonging cell survival rather than by accelerating rates of cellular proliferation. Specifically, *bcl-2* blocks programmed cell death, a physiological process that normally ensures a homeostatic balance between cell production and cell turnover in most tissues with self-renewal capacity and which often involves characteristic changes in cell morphology termed apoptosis. In fact, Bcl-2 can prevent or delay apoptosis induced by a wide variety of stimuli, including growth factor deprivation, alterations in Ca^{2+}, free radicals, cytotoxic lymphokines, some types of viruses, radiation, and most chemotherapeutic drugs, suggesting that this oncoprotein controls a common final pathway involved in cell death regulation (reviewed in refs. 1 and 2).

The mechanism by which Bcl-2 prevents cell death remains enigmatic, as the predicted amino acid sequence of the 26-kDa human Bcl-2 protein (239 aa) has no significant homology with other proteins whose biochemical activity is known. Recently, however, Bcl-2 has been shown to interact with a low molecular weight GTPase member of the Ras family, p23-R-Ras (3), and also can be coimmunoprecipitated with the serine/threonine-specific protein kinase Raf-1 (4). Thus, Bcl-2 may somehow regulate a signal transduction pathway involving R-Ras and Raf-1. In addition, p26-Bcl-2 has been shown to form heterodimers (or possibly hetero-oligomers) with a 21-kDa protein, Bax. The Bax-α protein has ≈21% amino acid identity with Bcl-2 and is topographically similar to Bcl-2 in that both proteins contain a stretch of hydrophobic amino acids near their C termini (5). Gene transfer studies in lymphokine-dependent hemopoietic cells indicate that Bax antagonizes Bcl-2 function, abrogating the ability of Bcl-2 to prolong cell survival in the setting of growth factor withdrawal (5). It is unclear, however, which of these two proteins, Bcl-2 or Bax, is the active effector and which is the regulator. In this regard, two non-mutually exclusive possibilities exist: (*i*) Bcl-2 could induce a pathway that actively maintains cell survival, with Bax serving as a negative regulator of Bcl-2, or (*ii*) Bax could directly or indirectly generate death signals, with Bcl-2 serving in this case as a dominant inhibitor of Bax.

In addition to *bax*, several other cellular genes have been reported that encode proteins which share sequence homology with Bcl-2. Among these is *bcl-X*, which can generate two proteins through an alternative splicing mechanism: Bcl-X-L (longer form), a 241-aa protein which has 43% sequence identity with Bcl-2 and which suppresses cell death, and Bcl-X-S (shorter form), a 178-aa protein that is missing a 63-aa region (aa 126–188) found in Bcl-X-L and which functions as a dominant inhibitor of Bcl-2 (6). Another of these Bcl-2 homologs is Mcl-1, which shares ≈35% sequence identity with Bcl-2 over a region of ≈140 aa (7). The function of Mcl-1 with regard to regulation of cell death has not been reported, nor has the issue of whether Bcl-X-L, Bcl-X-S, or Mcl-1 can interact with either Bcl-2 or Bax been addressed. Using a yeast two-hybrid system (8–10), we explored interactions among various Bcl-2 family proteins.

MATERIALS AND METHODS

Yeast Strains, Media, and Transformations. *Saccharomyces cerevisiae* strain EGY191 (*MATα trp1 ura3 his3 LEU2::pLexAop1–LEU2*) was grown in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose. Burkholder's minimal medium (11) fortified with appropriate amino acids was used for preparation of high-phosphate medium (0.15% KH_2PO_4) unless otherwise specified. Plasmid DNA transformations were done by the LiCl method (12); cells were grown in complete minimal medium lacking uracil,

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Abbreviation: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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tryptophan, or histidine as necessary to select for the presence of various plasmids. Yeast cell extracts were prepared by a spheroplast method (13), and production of LexA and B42 fusion proteins was verified by immunoblot assays using a LexA-specific polyclonal antiserum (14) or a hemagglutinin (HA1)-specific monoclonal antibody (clone 12CA5; Boehringer Mannheim).

Reverse Transcription–PCR Cloning of *mcl-1* cDNA. To obtain cDNAs for human *mcl-1*, total RNA from U-937 human monocytic cells was reverse-transcribed with recombinant Moloney murine leukemia virus reverse transcriptase (Superscript) (GIBCO/BRL) and a combination of random oligodeoxynucleotide hexamers and a *mcl-1*-specific primer complementary to a region 3′ of the open reading frame (5′-CATAATCCTCTTGCCACTTGC-3′). The first-strand cDNA was amplified by PCR using Vent polymerase (New England Biolabs), a 5′ primer containing a *Sac* I site (underlined) flanking the initiation codon (5′-CAGAGCTCGCAATGTTTGGCCTCA-3′), and a reverse primer complementary to sequences downstream of the *mcl-1* stop codon (5′-GAAGTTACAGCTTGGAGTCC-3′). The 1.1-kb PCR product was digested with *Sac* I and *Hin*cII and cloned into the plasmid pBluescript SKII (Stratagene). The *mcl-1* cDNA was confirmed by DNA sequencing to be free of errors that would alter the Mcl-1 protein.

Plasmid Constructions. The cDNAs encoding Mcl-1, mouse Bax (15), human Bcl-2 (16), human Bcl-X-L and Bcl-X-S (6), and human Fas/APO-1 (17) were modified by a PCR mutagenesis approach (18) for subcloning in-frame into the two-hybrid plasmids pEG202 (a derivative of pLex202-PL containing an expanded polylinker) and pJG4-5 (8–10). To avoid problems with targeting of proteins to the nucleus, sequences corresponding to the transmembrane domains of Bcl-2, Bcl-X-L, Bcl-X-S, Bax, and Mcl-1 were omitted and a stop codon was inserted. The pEG202 plasmid utilizes an *ADH* promoter to constitutively drive expression of fusion proteins containing an N-terminal LexA DNA-binding domain (aa 1–202). All cDNAs for testing were subcloned between the *Eco*RI and *Bam*HI sites of pEG202, in frame with the upstream *lexA* sequences. Forward and reverse primers (*Eco*RI site underlined, *Bcl* I site overlined, and stop codons in bold type) included (*i*) for Bcl-2 (aa 1–218), 5′-GGAATTCATGGCGCACGCTGGGAGAAC-3′ and 5′-TGATCACTTCAGAGACAGCCAC-3′; (*ii*) for Bcl-X-L (aa 1–212) and Bcl-X-S (aa 1–149), 5′-GGAATTCATGTCTCAGAGCAACCGG-3′ and 5′-CTGATCAGCGGTTGAAGCGTTCCTG-3′; (*iii*) for Bax (aa 1–171), 5′-GGAATTCGCGGTGATGGACGGGTCCGG-3′ and 5′-GGAATTCTCAGCCCATCTTCTTCCAGA-3′; (*iv*) for human Fas/APO-1 (aa 191–335), 5′-GGAATTCAAGAGAAAGGAAGTACAG-3′ and 5′-TGATCACTAGACCAAGCTTTGGAT-3′. (*v*) For Mcl-1 (aa 1–329), the PCR-generated "full-length" *mcl-1* cDNA described above was first subcloned into pUC18 to pick up an *Eco*RI site at the 5′ end and then was digested with *Xho* I and *Hin*cII, and the resulting 0.34-kb fragment representing the 3′ portion of the *mcl-1* open reading frame containing the transmembrane region was replaced with a 0.28-kb *Xho* I–*Sma* I fragment (lacking the transmembrane region) that was derived by reverse transcription–PCR from U-937 mRNA by using the primers 5′-AGAATTCACCTTACGACGGGTTGG-3′ and 5′-CGAATTCACCTGATGCCACCTTCTAG-3′. The resulting plasmid was digested with *Eco*RI, and the 1.0-kb fragment representing *mcl-1* (aa 1–329) was subcloned into the *Eco*RI sites of pEG202 and pJG4-5.

The pJG4-5 plasmid utilizes a galactose-dependent promoter from the *GAL1* gene to inducibly drive expression of fusion proteins containing an N-terminal B42 trans-activation domain, simian virus 40 nuclear localization signal sequence, and hemagglutinin (HA1) epitope tag (8–10). All cDNAs for testing were liberated from the pEG202 plasmids described above by cleavage with *Eco*RI and *Xho* I and then subcloned between the *Eco*RI and *Xho* I sites of pJG4-5, in frame with the upstream B42 sequences. The plasmids pEG202-c-raf-1, pEG202-laminC, and pJG4-5-laminC were constructed from pBMT116-c-raf-1 and pBMT116-laminC (19) generously provided by S. Hollenberg (University of Washington).

For construction of Bcl-2 deletion mutants, pEG202-Bcl-2-(1–81) and pJG4-5-Bcl-2-(1–81), the pEG202-Bcl-2 and pJG4-4-Bcl-2 plasmids were digested with *Sac* II/*Bam*HI and *Sac* II/*Xho* I, respectively. The ends of these digested plasmids were intramolecularly ligated after blunting with T4 DNA polymerase. The pEG202-Bcl-2-(1–81) and pJG4-5-Bcl-2-(1–81) plasmids contain an additional 3 codons and 38 codons of plasmid-derived coding sequences before the stop codon, respectively. For construction of pEG202-Bcl-2-(83–218) and pJG4-5-Bcl-2-(83–218) plasmids, both pEG202-Bcl-2 and pJG4-5-Bcl-2 were digested with *Sac* II and *Eco*RI, and the ends were made blunt with T4 DNA polymerase and then intramolecularly ligated. The structure of the regions of Bcl-2, Bax, Bcl-X, and Mcl-1 subcloned into yeast expression plasmids is diagrammed in Fig. 1. Proper construction of all plasmids and absence of PCR-generated errors were verified in every case by DNA sequence analysis.

***β*-Galactosidase Assays.** EGY191 cells were stably transformed with the LexA operator–*lacZ* reporter gene plasmid SH18-34 (gifts of S. Hanes, Massachusetts General Hospital, Boston) and selected for growth on uracil-deficient medium. For plate assays, yeast were spotted onto SD minimal medium plates lacking uracil, tryptophan, and histidine and containing 2% glucose or 2% galactose and the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (20). Filter assays were performed with X-Gal as a substrate, essentially as described (21), except that Pall nylon membranes were used instead of nitrocellulose filters. Z-buffer (13) containing X-Gal (25 μg/ml) in *N,N*,-dimethylformamide was used for measurements of β-galac-

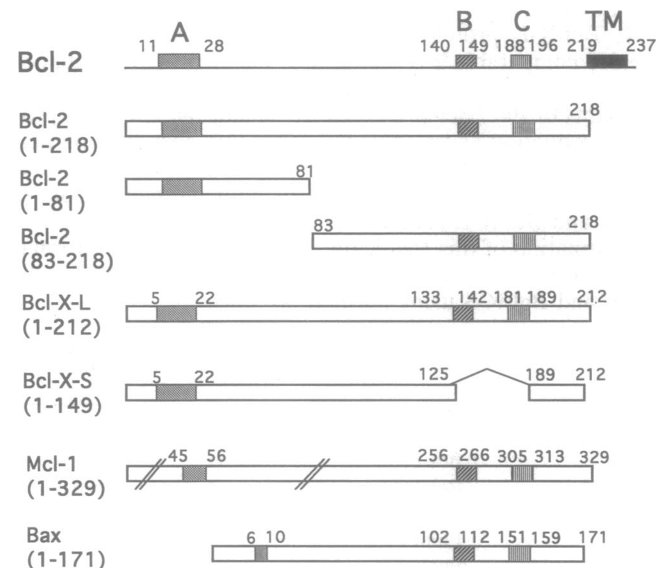

FIG. 1. Schematic depiction of regions of Bcl-2 and related proteins expressed as LexA or B42 fusion proteins. The structure of the human Bcl-2 protein is presented in linear form, indicating the three regions that are conserved among many of the family members (domains A, B, and C) and the corresponding amino acid positions. The transmembrane (TM) domain was excluded from all fusion proteins by introduction of a stop codon. The Bcl-X-S protein has a 63-aa deletion relative to Bcl-X-L because of a splicing event. The Mcl-1 protein is the longest of the family members (denoted by slashes).

tosidase activity. Filters were monitored for blue reaction products at 0.5, 1, 2, 4, and 24 hr.

RESULTS

Interactions Among Members of the Bcl-2 Protein Family. A LexA/Bcl-2 fusion protein containing aa 1–218 of the human Bcl-2 protein (lacking the C-terminal region, aa 219–239, which includes the transmembrane domain of Bcl-2) resulted in trans-activation of a *lacZ* reporter gene containing LexA operators when coexpressed with a fusion protein representing the B42 trans-activation domain fused with Bcl-2-(1–218) (Table 1). Furthermore, trans-activation of the *lacZ* reporter occurred only when cells were grown on galactose-containing plates but not on glucose plates, consistent with the galactose dependency of the *GAL1* promoter used to drive expression of the B42/Bcl-2-(1–218) expression plasmid. Expression of the LexA/Bcl-2 protein also resulted in *lacZ* reporter activation when combined with B42/Bcl-X-L, B42/Bcl-X-S, B42/Mcl-1, or B42/Bax protein in this assay (Table 1), implying that Bcl-2 can interact not only with itself but also with Bcl-X-L, Bcl-X-S, Bax, and Mcl-1. The interactions of Bcl-2 with itself and other members of the Bcl-2 family as revealed by the two-hybrid approach were specific, based on the use of control proteins including LexA/Raf, LexA/Fas, LexA/laminC, B42/Ha-Ras, and other B42 fusion proteins.

As with Bcl-2, expression of a LexA/Bcl-X-L fusion protein also resulted in *lacZ* reporter activation when combined with B42/Bcl-2, B42/Bcl-X-L, B42/Bcl-X-S, B42/Bax, or B42/Mcl-1 fusion protein but not with the B42/Ras, B42/5-1, or B42/5-2 protein (Table 1 and data not shown). Thus, Bcl-X-L appears to be capable of mediating the same spectrum of interactions among these members of the Bcl-2 protein family as that observed for Bcl-2. Interestingly, expression of a B42/Bcl-X-S protein in combination with either LexA/Bcl-2 or LexA/Bcl-X-L resulted in particularly high levels of *lacZ* reporter activation, as indicated by the more intense blue color produced in these colonies when grown on plates containing the β-galactosidase colorimetric substrate X-Gal, relative to cells expressing B42/Bcl-2 or B42/Bcl-X-L protein (Table 1). Immunoblot analysis revealed comparable levels of all B42 fusion proteins (data not shown), thus excluding quantitative differences in the amounts of B42/Bcl-X-S, B42/Bcl-2, and B42/Bcl-X-L protein production as an explanation for this phenomenon and suggesting that the interaction of Bcl-X-S with Bcl-2 and Bcl-X-L may be of higher affinity than the interactions of the Bcl-2 and Bcl-X-L proteins with themselves or each other. Confirmation of this suspicion, however, must await formal determination of the dissociation constants for Bcl-2 with itself and Bcl-X-S using purified proteins.

Structural Requirements for Bcl-2 Homodimerization. As a preliminary attempt to delineate the subregions within Bcl-2 that are required for Bcl-2/Bcl-2 homodimerization, constructs were prepared that encoded either LexA or B42 fusion proteins containing an N-terminal truncation mutant of Bcl-2 (aa 83–218). The LexA/Bcl-2-(83–218) protein interacted with a "full-length" B42/Bcl-2(1–218) protein but not the "truncated" B42/Bcl-2(83–218) fusion protein in the two-hybrid system (Table 2). Conversely, the B42/Bcl-2-(83–218) truncation mutant interacted with full-length LexA/Bcl-2-(1–218) but not with LexA/Bcl-2-(83–218). Taken together, these results exclude insufficient levels of B42/Bcl-2-(83–218) and LexA/Bcl-2-(83–218) protein production as an explanation for the failure of these N-terminal truncation mutants to interact with each other.

The finding that the Bcl-2 N-terminal deletion mutant was capable of mediating interactions with "full-length" Bcl-2-(1–218) but could not homodimerize with itself implies that amino acid sequences located between residues 1 and 82 of the Bcl-2 protein are required for interactions with the Bcl-2-(83–218) region. To test this hypothesis, a construct was prepared that encoded a B42 fusion protein containing aa 1–81 of human Bcl-2. The B42/Bcl-2-(1–81) protein interacted with LexA/Bcl-2-(1–218) and LexA/Bcl-2-(83–218) in the two-hybrid system, consistent with a model involving interaction of an N-terminal domain of Bcl-2 with the C-terminal region of Bcl-2 in a head-to-tail fashion. LexA/Bcl-2-(83–218) also resulted in *lacZ* reporter expression in a galactose-dependent manner when coexpressed with B42 fusion proteins containing Bcl-X-L, Bcl-X-S, or Mcl-1 (Table 2), implying that amino acid sequences sufficient for association with the 83–218 region of Bcl-2 are conserved within all of these proteins. The interactions mediated by the B42/Bcl-2-(1–81) protein appeared to be specific, since B42/Bcl-2-(1–81) failed to interact with LexA/laminC, LexA/CD40, LexA/Fas, and LexA/Raf negative control proteins (Table 2).

Bax Exhibits a Lethal Phenotype in Yeast That Is Neutralized by Bcl-2, Bcl-X-L, and Mcl-1. When Bax was expressed in yeast as a B42 fusion protein in pJG5.4 under the control of a galactose-inducible *GAL1* gene promoter, growth upon replica plating from glucose to galactose-containing plates was retarded in many cases (Tables 1 and 2). Moreover, expression of Bax as a LexA fusion protein in pEG202 under the control of the *ADH* promoter—a stronger, constitutive promoter—resulted in nearly complete absence of colony formation on glucose plates (Table 3). In contrast, transformation of cells with the same expression vector, pEG202, containing the *bax* cDNA in reverse (antisense) orientation produced colonies on glucose-containing medium at approx-

Table 1. Summary of yeast two-hybrid assay results for Bcl-2 and Bcl-X-L proteins

	B42							
LexA	Bcl-2	Bcl-X-L	Bcl-X-S	Mcl-1	Bax	Clone 1	Clone 2	[Val^{12}]Ras
Bcl-2	+	+	+++	+	+	−	−	−
Bcl-X-L	+	+	+++	+	+	−	−	−
c-Raf	−	−	−	−	−	−	−	++
Lamin	−	−	−	−	−	−	−	−
Fas	−	−	−	−	−	−	−	−

Versions of the pEG202 expression plasmids producing either LexA DNA-binding-domain fusion proteins (listed at left) or derivatives of pJG4-5 that encode B42 trans-activation-domain fusion proteins (listed at top) were introduced into EGY191 cells (5 μg each) and the resulting transformants were grown on plates containing X-Gal and either galactose or glucose as a carbon source. Combinations of two-hybrid plasmids that resulted in galactose-dependent production of blue colored colonies are indicated as +, ++, or +++ depending on the relative strength of the blue color reaction. All positive combinations produced discernible blue color within 4 hr in filter assays. Data were in agreement in all cases for both plate and filter assays. Some plasmid combinations failed to result in blue color production on either galactose or glucose-containing medium (−). Clones 1 and 2 are random cDNAs from a HeLa cell library (8).

Table 2. Analysis of interactions of Bcl-2 deletion mutants by two-hybrid assay

LexA	B42								
	Bcl-2	Bcl-2-(83–218)	Bcl-2-(1–81)	Bcl-X-L	Bcl-X-S	Bax	Mcl-1	Clone 1	Clone 2
Bcl-2	+	++	+	+	+++	+	+	–	–
Bcl-2-(83–218)	++	–	++	++	++	– (PG)	+	–	–
Bcl-X-L	+	++	+	+	+++	+	+	–	–
Fas	–	–	–	–	–	– (PG)	–	–	–
CD40	ND	ND	–	ND	ND	ND	ND	–	–
c-raf	–	–	–	–	–	– (PG)	–	–	–
Lamin	–	–	–	–	–	– (PG)	–	–	–

Experiments were performed as in Table 1. Transformants that grew poorly are indicated (PG). ND, not done.

imately normal efficiencies (Table 3). Additional experiments (data not shown) indicated that expression of the LexA/Bax protein was lethal to EGY191 yeast cells and not merely growth inhibitory.

In contrast, when EGY191 cells that had been cotransformed with the LexA/Bax vector and galactose-inducible B42/Bcl-2, B42/Bcl-X-L, or B42/Mcl-1 plasmid were plated directly on galactose-containing medium, colony formation occurred. Coexpression of B42/Bcl-X-S, however, did not nullify the inhibitory activity of LexA/Bax. The deletion mutant forms of Bcl-2, B42/Bcl-2-(1–81), and B42/Bcl-2-(83–218), as well as the control proteins B42/laminC and B42/5-1, also failed to abrogate the suppressive effects of LexA/Bax on colony formation (Table 3). Immunoblot analysis demonstrated comparable levels of all B42 fusion proteins in EGY191 cells (data not shown), suggesting that quantitative differences in the amounts of B42 fusion protein production cannot account for the failure of the B42/Bcl-X-S, B42/Bcl-2-(1–81), and B42/Bcl-2-(83–218) proteins to nullify the inhibitory effects of LexA/Bax.

DISCUSSION

Using a yeast two-hybrid system, we have obtained evidence for combinatorial interactions among several members of the Bcl-2 protein family. In addition to the previously reported interaction of Bcl-2 with Bax (5), the data presented here suggest that the following additional interactions can occur among Bcl-2 family proteins: (*i*) Bcl-2 with Bcl-2, (*ii*) Bcl-2 with Bcl-X-L, (*iii*) Bcl-2 with Bcl-X-S, (*iv*) Bcl-2 with Mcl-1, (*v*) Bcl-X-L with Bcl-X-L, (*vi*) Bcl-X-L with Bax, (*vii*) Bcl-X-L with Bcl-X-S, (*viii*) Bcl-X-L with Mcl-1, and (*ix*) Bax with Mcl-1. Of course, interpretation of results obtained by use of the two-hybrid approach must take into consideration several caveats. First, the results do not exclude the possibility that the interactions detected among members of the Bcl-2 family are indirect, requiring an additional bridging protein(s) that is conserved from yeast to humans. Second, the stoichiometry of these interactions can also not be inferred from these data, and while it is attractive to view the results as consistent with a homo/heterodimerization model, one cannot exclude other possibilities. Third, though the data presented here reveal the array of protein–protein interactions that are theoretically possible, they do not guarantee that these interactions occur in physiologically relevant contexts in mammalian cells.

The finding that LexA/Bax has an apparently lethal effect in *S. cerevisiae* strongly suggests that Bax is a cell-death effector protein, as opposed to merely a repressor of proteins (e.g., Bcl-2) that may be necessary for cell survival. However, it is possible that the lethal effects of Bax in yeast reflect its ability to bind to and neutralize an endogenous yeast homolog of Bcl-2—though we favor the alternative interpretation, given our previous lack of success with cloning of Bcl-2 homologs from yeast (unpublished data). While ectopic expression of mammalian proteins in yeast can sometimes be nonspecifically toxic, the lethal effect of Bax appeared to be specific, inasmuch as it was partially abrogated by Bcl-2 and Bcl-X-L, two known suppressors of apoptotic cell death, but not by Bcl-X-S, a dominant inhibitor of Bcl-2. To the extent that the cell-death pathways in mammalian cells and yeast are conserved, our discovery of an apparently lethal phenotype for Bax in *S. cerevisiae* suggests possibilities for using the power of yeast genetics to map this pathway and delineate several of the proteins involved.

Our preliminary attempts to map the regions within Bcl-2 that are necessary for homodimerization suggest a head-to-tail model for this protein–protein interaction (Fig. 2). This model is consistent with the observation that a LexA/Bcl-2-(83–218) fusion protein was able to complement a B42/Bcl-2-(1–81) fusion protein whereas it did not interact with a B42/Bcl-2-(83–218) protein. Unfortunately, it was not possible to perform the converse experiment using a LexA/Bcl-2-(1–81), because of nonspecific reporter gene activation. The explanation for this phenomenon is unclear, but the nonspecific activation could be due to a short stretch of acidic residues at positions 28–34 of the human Bcl-2 protein which may become unmasked in the deletion mutant LexA/Bcl-2-(1–81) and thus be able to function directly as a trans-activating sequence (10). When the Bcl-2-(1–81) region was expressed as B42 fusion, however, no problems with nonspecific reactivity were encountered. For example, while the B42/Bcl-2(1–81) protein complemented LexA/Bcl-2-(1–218) ("full-length" Bcl-2) and LexA/Bcl-2-(83–218) proteins in the two-hybrid system, it displayed absolutely no reactivity

Table 3. Neutralization of Bax activity by Bcl-2, Bcl-X-L, and Mcl-1

LexA	Medium	B42							
		Bcl-2	Bcl-X-L	Bcl-X-S	Mcl-1	Bcl-2-(83–218)	Bcl-2-(1–81)	Clone 1	Lamin
Bax (sense)	Gal	2.8×10^2	3.0×10^2	– (10)	2.9×10^2	– (16)	– (19)	– (9)	– (8)
	Glc	– (1)	– (5)	– (3)	– (1)	– (10)	– (10)	– (15)	– (7)
Bax (antisense)	Gal	1.2×10^3	ND	ND	ND	ND	ND	ND	2.2×10^3
	Glc	6.6×10^2	ND	ND	ND	ND	ND	ND	2.2×10^3

EYG191 cells were cotransformed with 5 μg each of pEG202- and pJG4-5-based expression plasmids and plated onto semisolid medium containing either galactose (Gal) or glucose (Glc). The number of visible colonies that formed is indicated; for plates scored as negative (–) for growth, the number of visible colonies is given in parentheses. ND, not done. Data are representative of three of three experiments.

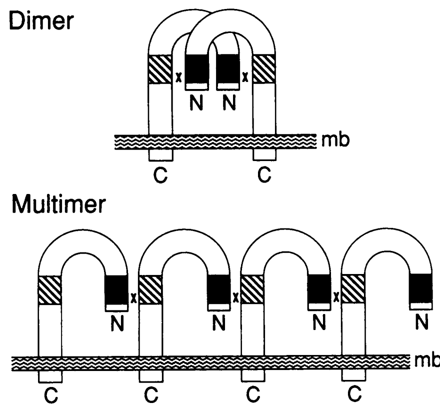

FIG. 2. Head-to-tail model for Bcl-2 protein interaction. The Bcl-2 protein contains a hydrophobic C-terminus that anchors it in membranes (mb). Interaction (X) between an N-terminal domain (black boxes) and a C-terminal domain (striped boxes) is required for formation of protein dimers or multimers.

with a variety of negative control LexA fusion proteins, including LexA/laminC, LexA/CD40, LexA/Raf-1, and LexA/Fas. Though our data are consistent with homodimerization of Bcl-2 proteins occurring via intermolecular interactions between two distinct regions within the protein, these results could instead reflect an intramolecular interaction of the 1–81 region of Bcl-2 with the 83–218 region. However, the ability of "full-length" Bcl-2-(1–218) fusion proteins to interact in the two-hybrid system with Bcl-2-(1–81) and Bcl-2-(83–218) fusion proteins tends to argue against this alternative interpretation. The precise amino acid residues within Bcl-2 involved in this multimerization are a matter for future investigations, but likely candidates are the three domains that are highly conserved among many members of this multigene family (22) [referred to as Bcl-2 domains (BD) A–C in Fig. 1]. Of note, however, is the finding that the Bcl-X-S protein, which lacks BD-B and BD-C because of an alternative splicing event, can still interact with Bcl-2 and Bcl-X-L in the two-hybrid system. This result suggests that BD-B and BD-C are not required for interaction of Bcl-X-S with Bcl-2 and Bcl-X-L and is consistent with the idea of a head-to-tail interaction where an N-terminal domain in Bcl-X-S can interact with a C-terminal domain or domains in Bcl-2 and Bcl-X-L—which could include, however, BD-B or BD-C within Bcl-2 and Bcl-X-L. It is interesting, therefore, that Bcl-X-S did not neutralize Bax-mediated cytotoxicity in yeast whereas Bcl-2 and Bcl-X-L did (Table 3). This result implies that the 63-aa region missing from Bcl-X-S, which contains BD-B and BD-C, is important either for heterodimerization of Bax with Bcl-2 and Bcl-X-L, or for neutralization of Bax function, or both.

Although the two-hybrid data indicate that both Bax and Bcl-X-S can bind to Bcl-2, our data derived from *S. cerevisiae* suggest that these dominant inhibitors abrogate Bcl-2 function through different mechanisms. For example, Bax was lethal to EGY191 cells when expressed at high levels as a fusion protein with LexA, whereas LexA/Bcl-X-S did not produce similar results and, in fact, LexA/Bcl-X-S-expressing cells appeared to have a slight growth advantage relative to other transformants (unpublished data). These results therefore suggest a model that proposes different mechanisms by which the dominant inhibitors Bax and Bcl-X-S abrogate Bcl-2 function. In this model (Fig. 3), Bax is envisioned as a cell-death effector whose activity is neutralized by binding of Bcl-2. The binding of Bcl-X-S to Bcl-2 we hypothesize prevents Bcl-2 from interacting with Bax and thus leaves Bax unopposed in its cell-death effector function. It may be of further relevance that the interaction of Bcl-X-S

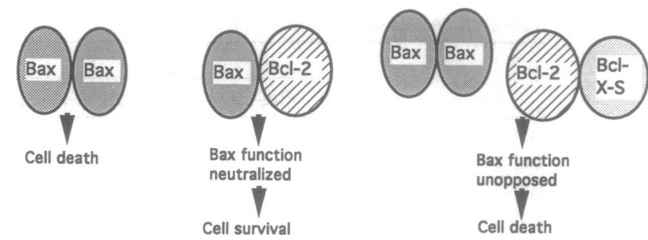

FIG. 3. Model for differential regulation of cell death by Bax and Bcl-X-S. In this speculative model, Bax is theorized to form a homodimer with itself that generates cell-death "signals" (*Left*). Heterodimerization of Bcl-2 and Bax abrogates Bax function (*Center*). Bcl-X-S binding to Bcl-2 prevents Bcl-2 from binding to and neutralizing Bax (*Right*).

with Bcl-2 in two-hybrid assays appeared to be more potent than the interaction of Bcl-2 with itself, suggesting that Bcl-X-S/Bcl-2 heterodimers represent higher-affinity protein–protein interactions than Bcl-2/Bcl-2 homodimers. Though highly speculative, this model provides a reasonable starting point for future investigations and is consistent with all of the data available to date.

Note Added in Proof. A recent paper by Yin *et al.*. (23) confirmed portions of this work in mammalian cells.

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