**Bcl-xS and Bad Potentiate the Death Suppressing Activities of Bcl-xL, Bcl-2, and A1 in Yeast**

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Members of the Bcl-2 family can be grouped into three classes based upon their effects on cell death. The first class suppresses death and includes Bcl-2. A second group, which includes Bax, is lethal, whereas a third class, including Bcl-xS, potentiates killing, although the members are not lethal by themselves. The proteins in the last class are proposed to exert their activity by binding to anti-apoptotic family members, thereby making the cell more susceptible to killing by another agent. To test this hypothesis, an inducible yeast expression system is reported that permits the functional analysis of three Bcl-2 family members. In yeast, Bax is lethal, and this activity is suppressed by Bcl-xL, Bcl-2, and A1. Co-expression of Bcl-xS did not diminish the ability of any of the anti-apoptotic members to antagonize Bax. Rather, co-expression of Bcl-xS potentiated the anti-death activity of all three proteins. This effect was not the result of changes in either the levels or integrity of Bax or anti-apoptotic proteins. Thus, Bcl-xS can bind to anti-apoptotic family members, but this association does not result in loss of biological activity. Therefore, Bcl-xS may act downstream of Bax and in a pathway that is conserved in yeast.

Regulated cell death is an essential and integral component of development, where it is used to eliminate transient structures, sculpt organs, and ablate supernumerary cells (1–3). In the adult, there is an exquisite balance between cell proliferation and cell elimination that maintains tissue homeostasis (1, 2). In addition, cell death in the adult serves to form certain tissues, as well as to eliminate self-reactive lymphocytes and virus-infected cells, and limit the life span of cells that may pose a threat to the organism through mutation or damage (1, 4, 5). Thus, perturbation of cell death can contribute to developmental defects, immune dysfunction, neurodegenerative disorders, and cancer (6). Therefore, the elucidation of the molecular mechanisms that control cell death is central to our understanding of an array of significant pathological conditions in man.

Cell death is a regulated process involving interactions among extracellular molecules, intracellular signal transduction pathways, and resident suicide/rescue programs (7–9). Pioneering work in *Caenorhabditis elegans* provided the fundamental molecular framework upon which cell death signaling/effector pathways in vertebrates have been modeled (10).

Bcl-2, the vertebrate homolog of *ced-9* in *C. elegans*, was the first mammalian gene shown to inhibit cell death in a variety of cell types and circumstances (11–15). Moreover, subsequent investigations identified a number of genes in vertebrates whose products were structurally and functionally related to Bcl-2 (8). These proteins constitute a family whose members share a number of regions of sequence homology (Bcl-2 homology domains, BH1, BH2, BH3, and BH4), and all variously influence cell death (16–18). Some of the Bcl-2-related proteins, such as Bcl-xL, also prevent cell death, whereas others, such as Bax, provoke cell elimination (19, 20). In addition, some members of this family, such as Bcl-xS and Bad, neither kill nor rescue cells per se, although they are able to promote cell death induced by other signals (19, 21).

The biochemical mechanisms that confer the death regulatory properties on the Bcl-2 family of proteins remain elusive, although recent structural data suggest that they may be pore-forming proteins (22). However, these proteins can participate in homo- and heteromeric complexes, and it has been suggested that their biological activities are determined by their spectrum of intra-family protein-protein associations (23–25). For example, Bcl-xL is suggested to suppress cell death by binding to the lethal family members, thereby neutralizing their activity. On the other hand, Bcl-xS is suggested to potentiate killing by selectively binding to the anti-apoptotic (but not lethal) members, effectively sequestering them and sensitizing the cell to the lethal effects of other agents (24, 25). However, several studies have shown that some viral as well as mutated versions of anti-apoptotic members of the Bcl-2 family fail to associate with lethal family members yet still suppress killing (26–28). Moreover, although Bcl-xS can bind to Bcl-xL, it is unclear whether this binding is of sufficient avidity to effectively displace it from Bax (29). Thus, protein-protein association may not be the sole determinant of biological activity. In particular, these results led us to question whether family members such as Bcl-xS potentiate killing by binding to, and thereby neutralizing, anti-apoptotic members such as Bcl-2 and Bcl-xL.

In prior studies, it was established that Bax and Bak are lethal when expressed in yeast (27, 30–32). In addition, co-expression of anti-apoptotic family members such as Bcl-2 or Bcl-xL suppressed killing induced by both Bax and Bak (27, 30). This provided the basis of an assay to determine whether death potentiating Bcl-2 family members, such as Bcl-xS and Bad, could antagonize the death suppressive effects of the anti-apoptotic members with Bax as the lethal agent. Using an inducible triple expression assay in yeast, it is shown here that rather than antagonize anti-apoptotic family members, Bcl-xS and Bad potentiate their ability to suppress Bax-mediated killing.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**As previously reported, the LexA fusions of Bcl-2, Bcl-xL, Bcl-xS, Bad, and A1 were made in the yeast expression plasmid YLexA (LexA), which carries the *Saccharomyces cerevisiae TRP1* gene as a
selectable marker (27, 33). These Bcl-2 family members are fused to the LexA operator-binding domain of the LexA protein. Fusion proteins with the transcriptional activation domain of the Herpes simplex virus protein VP16 were constructed in the plasmid pSD.10a (VP16), which harbors the URA3 gene as a selectable marker (34). Unfused Bad was generated in Y.LexA by deletion of the LexA codons. Other unfused proteins were constructed in the plasmid pSD.10a (VP16), which carries the TRP1 selectable marker (34). One of the colonies was named S50-bax and used in the experiments reported here.

Yeast growth and maintenance was according to the standard protocols (36). Transformations were performed by the lithium acetate method as described (37).

Yeast Growth Assay—Yeast S50-bax was (co)transformed with expression plasmids encoding Bcl-2 family members, or parental control vectors, VP16 (which carries the URA3 selectable marker) or LexA (which carries the TRP1 selectable marker). A yeast growth assay was performed as described previously (27). Typically 10 ml of selective medium (with 2% galactose) was inoculated with 1.5 × 10⁶ cells and then incubated at 30 °C. Samples were taken at different time points, and cell density was measured by determining the OD₆₆₀. To compare results from different experiments, a growth index (GI) was used:

\[ \text{GI} = \frac{\text{OD}_{660/22 \text{~h}} - \text{OD}_{660/0 \text{~h}}}{\text{cells expressing Bax and the test proteins}} \]

(1)

Immunoblotting—Yeast cells were lysed mechanically as described (38), and the protein content of the lysate was determined by the Coomassie Brilliant Blue G-250 dye method (Bio-Rad). Proteins were separated on 15% SDSPolyacrylamide gels (50 μg/lane) and transferred to nitrocellulose membranes. Preblocking and immunostaining of blots were performed in Tris-buffered saline containing 1% Tween 20 and 5% nonfat milk at room temperature. After preblocking for 1 h, membranes were incubated with a rabbit anti-murine Bax polyclonal antibody, 13686E (1:1000 dilution) (PharMingen), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibacteriol (1:1000) (Amersham Pharmacia Biotech), both for 2 h. Immunoblots were developed using the enhanced chemiluminescence method (Pierce). For Bcl-2 and Bcl-x immunostaining, the membrane was stripped of bound antibody by incubating the strip in a buffer comprising 62 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 20 min and then reprobing with a monoclonal anti-Bcl-2 antibody, 15021A (1:250) (PharMingen), or a monoclonal anti-Bcl-x antibody, B22620 (1:250) (Transduction Laboratories).

RESULTS

To investigate the functional interactions among lethal, pro-apoptotic, and anti-apoptotic members of the Bcl-2 family, an inducible triple expression assay was developed in S. cerevisiae. Because Bax is lethal in yeast (27, 30, 32), this Bcl-2 family member was selected as the killing agent. A murine bax gene was stably integrated into the genome of the S50 yeast strain where it was under the control of the GAL10-CYC1 hybrid promoter, making it galactose-inducible and glucose-repressible. This yeast strain is referred to as S50-bax. When cultured in glucose, basal levels of Bax were undetectable by immunoblotting in S50-bax (Fig. 1A), and the cells grew robustly (data not shown). However, upon switching from glucose to galactose, Bax expression was induced (Fig. 1A), and the yeast died (Fig. 2). The plasmid pSD-Bcl-xL encodes full-length murine Bcl-xL and is galactose-inducible (27). Transformation of S50-bax with the pSD-Bcl-xL plasmid resulted in a nearly complete reversal of Bax lethality when the yeast were grown in galactose (Fig. 2A). These data in S50-bax recapitulated results gained in S260 yeast when both Bax and Bcl-xL were co-expressed from plasmids (27). This system offered the opportunity to co-express pro-apoptotic Bcl-2 family members with Bax and Bcl-xL. Bcl-xS is a splice variant of Bcl-xL that has pro-apoptotic activity in mammalian cells (19). The differential splicing in Bcl-xS eliminates the BH1 and BH2 domains present in Bcl-xL and results in the loss of Bax binding and the conversion from an anti- to a pro-apoptotic protein (19, 24, 25, 27). Three properties of Bcl-xS make it the best choice for the pro-apoptotic...
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**FIG. 2.** Bcl-xS does not antagonize the death suppressing activity of Bcl-xL in Bax-expressing yeast. A, yeast strain S50-bax was transformed with the parental control vector (VP16) or a plasmid encoding Bcl-xL or LexA-Bcl-xS or cotransformed with plasmids encoding Bcl-xL and LexA-Bcl-xS. Expression of Bax from the integrated transgene in S50-bax is indicated by (Bax). Cell density was determined at various time intervals by measuring OD_{660}. B, S50-bax were transformed with the parental control vector (LexA) or a plasmid encoding LexA-Bcl-xL or Bcl-xS or cotransformed with plasmids encoding LexA-Bcl-xL and Bcl-xS. Growth was assessed at various times by measuring OD_{660}. The data shown are representative of three experiments.

component in the present analysis. First, Bcl-xS has been shown to antagonize the anti-apoptotic activity of Bcl-xL in vertebrate cells (29). Second, Bcl-xS binds to Bcl-xL but not Bax in yeast (24, 25, 27). Third, expression of Bcl-xS alone does not affect yeast viability (27) avoiding complications in data interpretation. Therefore, Bcl-xS would be predicted to antagonize the death suppressing activity of Bcl-xL in yeast.

The pSD-Bcl-xS plasmid encodes murine Bcl-xS under the control of a GAL10-CYC1 promoter. Co-expression of Bcl-xS with Bcl-xL and Bax did not reduce yeast growth or viability (Fig. 2). Rather, expression of Bcl-xS consistently conferred a slight growth advantage to both S50-bax and S50-bax co-expressing a LexA fusion of Bcl-xL (Fig. 2B). Thus, despite the fact that Bcl-xS dimerizes with Bcl-xL in yeast (27), this interaction did not attenuate the anti-apoptotic activity of the latter. Indeed, the data suggest that Bcl-xS may potentiate the activity of Bcl-xL. However, because Bcl-xL almost completely reversed Bax lethality (27) (Fig. 2), it was difficult to assess whether Bcl-xS truly augmented anti-apoptotic activity. In addition, the data raise a number of concerns in interpretation. First, Bcl-xS may have some selectivity in which anti-apoptotic members it can suppress (although Bcl-xS can specifically antagonize Bcl-xL in vertebrate cells) (29). Second, the present assay imposes an upper limit on rescue that could mask positive or negative effects of additional agents, particularly if Bcl-xL were especially potent and required only at low concentrations to suppress Bax. Therefore, further experiments were performed to clarify the biological properties of Bcl-xS in yeast.

It was noted previously that both the lethal and anti-apoptotic members of the Bcl-2 family have different potencies in yeast (27). Moreover, the presence of additional amino acids in some Bcl-2 family members attenuates their activity (27). For example, the LexA fusion of Bcl-xL is less potent than the unfused protein (Fig. 2). This situation provided the opportunity to analyze the functional interactions between Bcl-xS and other, less potent anti-apoptotic family members and their derivative fusion proteins in S50-bax.

As shown in Table I, a number of anti-apoptotic Bcl-2 family members all suppressed Bax lethality to varying degrees in S50-bax. The rank order of potency of these proteins against Bax lethality was Bcl-xL > LexA-Bcl-xL > LexA-A1 > Bcl-2 > LexA-Bcl-2 > VP16-A1 (Table I). In no case did Bcl-xS attenuate the anti-apoptotic activity of these proteins, despite the fact that it binds to all of them in the yeast two-hybrid assay (27). Thus, there is no evidence for selective suppression of any

### Table I

| Transgene | Plasmid 1 | Plasmid 2 | GI |
|-----------|-----------|-----------|----|
| Bax       | VP16      | LexA      | 1.0|
| Bax       | Bcl-xS    | LexA      | 1.0|
| Bax       | Bcl-xL    | LexA      | 20.9|
| Bax       | Bcl-xL    | LexA-Bcl-xS | 22.5|
| Bax       | Bcl-2     | LexA      | 16.2|
| Bax       | Bcl-2     | LexA-Bcl-xS | 21.6|
| Bax       | VP16-A1   | LexA      | 10.0|
| Bax       | VP16-A1   | LexA-Bcl-xS | 16.8|
| Bax       | LexA-Bcl-xS | VP16      | 1.0|
| Bax       | LexA-Bcl-xL | VP16      | 17.2|
| Bax       | LexA-Bcl-xL | Bcl-xS   | 18.9|
| Bax       | LexA-Bcl-2 | VP16      | 12.2|
| Bax       | LexA-Bcl-2 | Bcl-xS   | 18.8|
| Bax       | LexA-A1   | VP16      | 16.3|
| Bax       | LexA-A1   | Bcl-xS   | 16.9|
anti-apoptotic family member by Bcl-xS. On the other hand, co-expression of Bcl-xS enhanced the suppression of Bax by all anti-apoptotic proteins (Table I). This augmentation was most pronounced for the less potent anti-apoptotic members such as VP16-A1 (Table I and Fig. 3). For example, when expressed in S50-bax, VP16-A1 produced a GI (an index of rescue, see "Experimental Procedures") of 10.0 ± 1.3 (mean ± S.D., n = 6). However, when VP16-A1 was co-expressed with Bcl-xS the GI was 16.8 ± 1.6 (mean ± S.D., n = 6), a significant increase (p < 0.01, Wilcoxon-Mann-Whitney test). Thus, the presence of Bcl-xS produced a 68% increase in the growth index for VP16-A1 and made it as potent as Bcl-2 (Table I). These data establish that not only does Bcl-xS fail to suppress any of the anti-apoptotic family members tested but also that it leads to a potentiation of their activity.

Because Bcl-xS is invariably pro-apoptotic in vertebrate cells (19, 29), explanations for rescue effects of Bcl-xS are on the one hand that it reduces Bax expression or promotes its degradation or on the other hand that it promotes the expression or stability of the anti-apoptotic members. These possibilities were assessed by determining steady state protein levels by immunoblotting. Neither Bax levels nor its processing were overtly influenced by the presence of Bcl-xS or the other anti-apoptotic proteins tested (Fig. 1). Similarly, neither the levels nor processing of the anti-apoptotic members, Bcl-2, Bcl-xL (Fig. 1A), LexA-Bcl-2, and LexA-Bcl-xL (Fig. 1B) were detectably altered by co-expression of Bcl-xS. Therefore, it is concluded that Bcl-xS does not function by changing the expression or degradation of either Bax or the anti-apoptotic proteins.

Finally, we addressed the issue of whether the action of Bcl-xS was unique to this member of the pro-apoptotic subclass of the Bcl-2 family. Bad, like Bcl-xS, binds to anti-apoptotic members of the Bcl-2 family but not to Bax and potentiates killings (21). In yeast, Bad does not bind to Bax and is not lethal per se (27). Therefore, Bad was used in place of Bcl-xS in the triple expression assay. Like Bcl-xS, Bad did not potentiate the activity of Bcl-2, Bcl-xL, or A1 (Table II). Rather, Bad potentiated all three anti-apoptotic family members (Table II). Thus, two structurally distinct members of the pro-apoptotic class inhibit Bax activity in yeast, although they do not associate with it.

**DISCUSSION**

The finding that Bcl-xS and Bad antagonize the activity of Bax without binding to it provides additional evidence for the emerging view that the association of anti-apoptotic family members with lethal members is not essential for death suppression (26–28). For example, in yeast, there is no requirement for Bcl-xL to bind to Bax to inhibit its lethality (27). This indicates that Bcl-xL can act downstream of Bax, perhaps by competing for binding to a common target protein. Because Bcl-xS and Bad do not bind to Bax, they too may interact with this hypothetical target protein, either independently or in association with an anti-apoptotic member.

To date, only the F$_{0}$F$_{1}$-ATPase has been implicated in the action of any Bcl-2 protein in yeast (39). However, in metazoans, downstream proteins include Ced-4 and Apaf-1, both putative ATP-binding proteins that variously interact with caspases and Bcl-2 family members to regulate cell death (40, 41). Because yeast do not exhibit programmed cell death and do not express classical caspases or Bcl-2 family members (27), it is probable that the hypothetical target protein serves another function in yeast. Also the absence of death effector molecules, such as caspases in yeast (27), may account for the paradoxical properties of Bcl-xS in this organism. Nevertheless, the fact that mammalian proteins such as Bax, Bcl-xL, and Bcl-xS are active in yeast indicates that the downstream mechanisms through which they act are present in lower eukaryotes and may have been adapted for cell elimination with the evolution of the multicellular state. The ability to manipulate the genome of yeast makes it an ideal organism in which to identify these evolutionarily conserved pathways.

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