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13. ABSTRACT (Maximum 200 words) Defects in the regulation of programmed cell death (apoptosis) play an important role in the pathogenesis of breast cancer, allowing neoplastic cells to survive far longer than their normal counterparts, and thus providing a mechanism for clonal expansion independent of increased cell division. Moreover, failures in normal apoptosis pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations, promoting resistance to immune-based destruction, allowing disobedience of cell cycle checkpoints which would normally induce apoptosis, facilitating growth factor/hormone-independent cell survival, allowing cell survival when detached from extracellular matrix during metastasis, reducing dependence on oxygen and nutrients, and conferring resistance to cytotoxic anticancer drugs and radiation. We discovered that expression of the proapoptosis gene <i>BAX</i> is lost in ~one-third of breast cancers, correlating with poor responses to chemotherapy and shorter survival for patients with metastatic disease. We also found that <i>BAX</i> gene transfer induced cell death not only in breast cancer cells but also yeast. Using yeast genetics approaches, we screened tumor cDNA libraries for suppressors of Bax, resulting in the discovery of a novel human Bax suppressor, BI-1. This anti-apoptotic protein thus becomes a new potential drug-discovery target for inducing apoptosis in cancers.					
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FOREWORD

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INTRODUCTION

The goal of the project was to understand more about how Bax functions. Bax is a pro-apoptotic protein which plays an important role in tumor responses to chemotherapy and radiation. This protein shares structural homology with the pore-forming domains of certain bacterial toxins, suggesting it may promote cell death through a pore- or channel-based mechanism.

The Bax protein is expressed in normal mammary epithelium. Our laboratory had shown that Bax expression is lost in about one-third of breast cancers (1, 2). In women with metastatic breast cancer, loss of Bax expression is associated with poor responses to chemotherapy and shorter overall survival (1).

We had discovered that Bax not only kills human cells but when expressed in yeast, it similarly conferred a lethal phenotype (3, 4).

We, therefore, sought to exploit the power of yeast genetics to identify downstream effectors or upstream regulators of Bax, in hopes that this information would permit us to develop strategies for restoring the Bax pathway for cell death in breast cancers.

RESULTS

Five specific tasks were proposed and work was performed on all of them, resulting in successful completion of 4 of 5.

Task #1. Use yeast two-hybrid library screening methods to identify yeast proteins that bind to Bcl-2 and Bax, particularly asking whether yeast contain homologs of Bcl-2 and Bax.

Yeast libraries were screened by the two-hybrid method in attempts to identify yeast proteins that bind to either Bcl-2 or Bax. None were found.

Task #2. Clone yeast and mammalian genes that can suppress the lethal phenotype of Bax in yeast.

Human tumor cDNA libraries were screened for suppressor of Bax. A novel human gene was thus discovered, which we termed Bax-Inhibitor-1 (BI-1). The predicted BI-1 protein contains at least 6 apparent transmembrane domains which share some similarity to ion-channels. The BI-1 protein is evolutionarily conserved, with close homologs in rats, mice, *C. elegans*, and plants, implying an important function. Immunolocalization experiments revealed that BI-1 is primarily located in the endoplasmic reticulum. BI-1 forms complexes with the anti-apoptosis proteins Bcl-2 and Bcl-XL, in co-immunoprecipitation and crosslinking experiments. When over-expressed in human epithelial cancer cell lines, BI-1 suppressed apoptosis induced by several stimuli. Conversely, antisense BI-1 induced apoptosis in these cells.

The findings thus establish BI-1 as a novel anti-apoptotic protein. A detailed report of the cloning and functional characterization of BI-1 was published in (5) (reprint attached). Methods, results and figures with legends follow below:

cDNA Cloning of Human BI-1

A yeast strain (QX95001) was constructed by transforming the Bax-expression plasmid YEp51-Bax, encoding the full-length mouse Bax protein under the control of the galactose-inducible yeast *GAL10* promoter (Zha et al., 1996), into strain BF264-15Dau (Lew et al., 1991). These yeast cells died upon transfer from glucose to galactose-containing medium, which induces the *GAL10* promoter in this plasmid and leads to accumulation of Bax protein in yeast (Zha et al., 1996). A human HepG2 cDNA expression library (Lew et al., 1991) was transformed into the QX95001 strain and screened for Bax-resistant transformants by plating on galactose-containing solid medium. From a screen of $\sim 10^6$ transformants, 17 displayed Bax resistance. Of these, 4 were determined to be dependent on the introduced cDNA-library plasmid by "con-committant-loss" assay (Ausubel et al., 1991). The nucleotide sequences of 3 of these cDNA clones encoded the same protein, designated

BI-1, for Bax Inhibitor-1. BI-1 did not interfere with production of the Bax protein in yeast, as determined by immunoblot analysis (Figure 1).

The Predicted BI-1 Protein Contains Several Membrane-Spanning Segments

All three BI-1 cDNAs obtained by the functional yeast screen contained an open reading frame (ORF) encoding a predicted protein of 237 amino acids. The predicted AUG start codon for this ORF was within a favorable context for translation initiation (Kozak, 1997) and was preceded by an in-frame stop codon. A search against the available nucleotide sequence databases using the BLAST program (Altschul et al., 1990) revealed that BI-1 is essentially identical to TEGT ("testis enhanced gene transcript"), a cDNA previously cloned fortuitously during an attempt to identify testis-specific genes (Walter et al., 1995). The rat TEGT protein has also been described and shares 90% identity (95% similarity) with the human protein (Walter et al., 1994). Additional previously undescribed homologs of BI-1 were also identified by BLAST searches in mouse, and possibly in the nematode *C. elegans* and the plant *Arabidopsis thaliana*. The mouse BI-1 protein, as deduced from ESTs #AA015124, AA275830, AA467259, AA107704, and W59401 is 237 amino acids in length and shares 92% identity (95% similarity) with the human BI-1 protein (Figure 2). The *C. elegans* ORF (EMBL #Q20241) encodes a protein of 241 amino acids that shares 21% overall amino acid identity (37% similarity) with human BI-1. This percentage homology shared by the nematode and mammalian BI-1 is similar to that reported for Ced-9 and Bcl-2,

which share only 23% identity in their amino acid sequences. The putative *Arabidopsis* homolog (EMBL #Z97343) is 261 residues in length, sharing 29% amino acid sequence identity (45% similarity) with the human BI-1 protein.

Based on Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982), the BI-1 protein is predicted to contain 6 or 7 transmembrane (TM) domains. The C termini of the mammalian BI-1 proteins are comprised of basic amino acids resembling some nuclear targeting sequences (Dingwall and Laskey, 1991), but otherwise the predicted proteins lack motifs that suggest a function. Of note, BI-1 does not contain any of the conserved BH domains of Bcl-2 family proteins. By searching a transmembrane protein database (TMbase) using the TMPred program (Hofmann and Stoffel, 1993), the most favored membrane topology for mammalian BI-1 is with 6 membrane-spanning domains and both the N and C termini oriented toward the cytosol. The putative transmembrane segments are predicted to assume mostly an α -helical conformation. Triton X-114 partitioning studies confirmed that BI-1 is an integral membrane protein (data not presented). RNA blot analysis indicated that BI-1 is widely expressed in vivo, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (not shown).

BI-1 Inhibits Bax-Induced Apoptosis in Mammalian Cells

Transient transfection of Bax-encoding expression plasmids induces apoptosis in the human embryonic kidney cell line 293 (Zha et al., 1996). Consequently, pcDNA3-hBax was cotransfected with equal amounts of pcDNA3 parental vector (used as a negative control), pcDNA3-BI-1-HA (HA-tagged BI-1), or pRc/CMV-Bcl-2 (used as a positive control). One day later, both the floating and adherent cells were collected and subjected to the trypan-blue vital dye exclusion assay. BI-1 suppressed Bax-induced cell death in 293 cells to a similar extent as Bcl-2 (Figure 3A). DAPI staining of 293 cell nuclei confirmed that Bax-induced cell death occurred by apoptosis (not shown). Immunoblot analysis demonstrated that BI-1 does not interfere with Bax production in 293 cells (Figure 3B).

BI-1 Inhibits Apoptosis Induced by Growth Factor Deprivation, Etoposide, and Staurosporine, but Not Fas

The effects of BI-1 overexpression on apoptosis induced by a variety of stimuli were explored. For many of these experiments, we employed a green fluorescent protein (GFP)-tagged BI-1, because it provided a convenient marker for transfected cells and because BI-1-GFP accumulated to higher levels than the BI-1-HA protein (not shown). Transient transfection studies in 293 cells confirmed that the BI-1-GFP protein retained biological activity as a suppressor of Bax-induced cell death (Figure 3C).

In the human diploid fibroblast line GM701, $\sim 30\%$ of the cells transfected with control GFP-marker plasmid

developed morphological characteristics typical of apoptosis when deprived of serum for ~30 hr. In contrast, GM701 cells transfected with plasmids encoding BI-1-GFP or Bcl-2 were substantially more resistant to serum deprivation, with only ~10% of the cells undergoing apoptosis (Figure 3D). Thus, BI-1 is able to suppress apoptosis induced by growth factor withdrawal in GM701 fibroblasts.

In many types of cells, apoptosis induced by the TNF-family receptor Fas (CD95) is poorly abrogated by Bcl-2 (reviewed by Vaux and Strasser, 1996). We therefore compared the effects of Bcl-2 with BI-1 in 293 cells where apoptosis was induced by transfection of Fas. Cotransfection of either BI-1 or Bcl-2 with a Fas-encoding plasmid failed to prevent apoptosis (Figure 3E). In contrast, Fas-induced cell death was markedly suppressed by cotransfection of a plasmid encoding XIAP, a protein that directly binds to and inhibits caspases required for Fas-induced apoptosis (Deveraux et al., 1997).

To further explore the role of BI-1 as an apoptosis inhibitor, we examined its effects in FL5.12, an interleukin-3-dependent pro-B lymphocyte clone previously shown to undergo apoptosis when deprived of IL-3. FL5.12 cells were stably transfected with plasmids encoding BI-1-GFP or Bcl-2 (as a positive control) and subclones obtained that expressed BI-1 or Bcl-2 at high levels. BI-1 transfectants were uniformly more resistant to apoptosis induction by IL-3 deprivation, with the extent of protection correlating roughly with the levels of BI-1-GFP protein. Figure 4A presents results from two clones, one with only modest and another with high levels of BI-1-GFP protein production. Note that even clones with low levels of BI-1-GFP protein exhibited some resistance to IL-3 deprivation, whereas clones expressing high levels of BI-1-GFP protein displayed pronounced resistance to apoptosis induction by IL-3 withdrawal, with the prolongation in cell survival approaching that seen in Bcl-2-transfected FL5.12 cells. BI-1 overexpression also afforded protection in FL5.12 cells against apoptosis induced by etoposide and staurosporine. Comparisons of FL5.12 cell clones with various levels of BI-GFP expression suggested that BI-1 was generally somewhat less effective than Bcl-2 at conferring resistance against these agents (Figures 4C and 4D, and data not shown).

Taken together, the transfection experiments in mammalian cells (Figures 3 and 4) demonstrate that BI-1 has antiapoptotic activity in epithelial, fibroblastic, and hematopoietic cells, and suggest that BI-1 shares functional similarity with Bcl-2 in delaying cell death induced by Bax, growth factor deprivation, staurosporine, and etoposide, but not Fas.

BI-1 Antisense Induces Apoptosis

A BI-1 cDNA was subcloned into pGL-Neo in reversed orientation and transiently transfected into 293 cells,

together with a GFP-marker plasmid. The BI-1 antisense (AS) plasmid induced apoptosis of 293 cells in a concentration-dependent manner, whereas control plasmid had no effect (Figure 5A). Lacking antibodies for detection of endogenous BI-1 protein to verify antisense-mediated down-regulation of BI-1 protein, parallel experiments were performed in which 293 cells were cotransfected with plasmids encoding BI-1-Flag protein and the BI-1-AS plasmid. As shown in Figure 5B, the levels of BI-1-Flag protein were markedly decreased in 293 cells that received the BI-1-AS plasmid compared to control transfected cells, as determined by immunoblotting. In contrast, the BI-1-AS plasmid had no effect on the levels of tubulin or other proteins examined, confirming the specificity of the results. These antisense experiments provide further evidence that BI-1 regulates apoptosis in mammalian cells.

BI-1 Is Located in Intracellular Membranes Similar to Bcl-2

The Bcl-2 and Bcl-X_L proteins are associated with intracellular membranes, primarily the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (Krajewski et al., 1993; González-García et al., 1994), while Bax appears to be localized mostly to mitochondria (Zha et al., 1996). To preliminarily explore the intracellular locations of the BI-1 protein, GFP-BI-1 protein was expressed in several different adherent cell lines (293, Cos-7, GM701). In all three cell lines, fluorescence microscopy demonstrated that BI-1 is exclusively cytosolic and appears to be associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (Figure 6A and data not shown). In contrast, GFP control protein was diffusely distributed throughout the cells. Only a small portion of BI-1 appears to be associated with mitochondrial membranes, based on two-color analysis using a mitochondria-specific fluorescent dye (Figure 6B). Similar results were obtained using a Flag-tagged BI-1 protein instead of GFP-BI-1 (Figure 6Ac).

The intracellular location of BI-1 was also explored by subcellular fractionation experiments. For this purpose, 293T cells were transiently transfected with the BI-1-HA-encoding plasmid or vector control. Cells were lysed in hypotonic buffer 2 days later and separated into crude subcellular fractions of nuclei (N), heavy membranes (HM), light membranes (LM), and cytosol (C) by differential centrifugation as described (Wang et al., 1996). As shown in Figure 6C, BI-1 was found mostly in the HM and N fractions (inferred as nuclear envelope based on the GFP fusion localization studies), as determined by immunoblot analysis where the fractions were normalized for cell equivalents. A small proportion of the BI-1-HA protein was also found in the LM fraction. The HM fraction contains mitochondria, lysosomes, and rough ER, whereas the LM fraction contains smooth ER, endosomes, and plasma membranes. As a control for the fractionation procedure, the same blot was reprobed with antibodies specific for the mitochondrial inner membrane protein F₁-β-ATPase, the nuclear protein PARP, and the cytosolic protein CPP32 (caspase-3). Bcl-2 was found essentially in the same subcellular compartments as BI-1, with most of this protein associated with the HM and N fractions (Figure 6C). We conclude therefore that BI-1 is associated with intracellular membranes, based on GFP-tagging experiments, immunofluorescence microscopy, and subcellular fractionation studies.

Recently, it was reported that a subpopulation of Bax molecules in cells are not integrated into membranes, but rather are found within a soluble cytosolic fraction, with the relative proportion of membrane-associated Bax increasing after application of apoptotic stimuli (Hsu et al., 1997). Comparisons of BI-1- and control-transfected 293T cells, however, demonstrated that BI-1 does not substantially change the relative amounts of Bax protein associated with various subcellular compartments (Figure 6C).

BI-1 Associates with Bcl-2 In Vivo

The subcellular fractionation data suggest that BI-1 and Bcl-2 colocalize to the same intracellular membranes. To address the question of whether BI-1 and Bcl-2 physically associate in membranes, we performed in vivo cross-linking experiments. Plasmids encoding either Flag-tagged or HA-tagged BI-1 were cotransfected with Bcl-2 into 293 cells. Cells were then incubated 2 days later with the thiol-cleavable chemical cross-linker DTBP. As shown in Figure 7A, both the HA- and Flag-tagged BI-1 proteins could be cross-linked to Bcl-2, suggesting that BI-1 and Bcl-2 come within close proximity to each other in membranes. In contrast, Bax was not cross-linked to BI-1 (data not shown).

To further explore the interaction of BI-1 with Bcl-2 family proteins, we attempted to coimmunoprecipitate BI-1 with Bcl-2, Bcl-X_L, Bax, and Bak. For these experiments, 293 cells, which contain high levels of Bak but very little endogenous Bcl-2, Bcl-X_L, or Bax, were transfected with Bcl-2, Bcl-X_L, or Bax-expression plasmids and either Flag-tagged BI-1 plasmid or empty vector. As shown in Figure 7B, Flag-tagged BI-1 protein specifically coimmunoprecipitated with Bcl-2, and Bcl-X_L but not Bax or Bak. Testing of several deletion mutants of Bcl-2 revealed that the BH4 domain is required for interactions with BI-1 (not shown). This domain is uniquely found in antiapoptotic but not most proapoptotic members of the Bcl-2 family (Reed, 1997a), presumably explaining why Bcl-2 and Bcl-X_L but not Bax and Bak form complexes with BI-1.

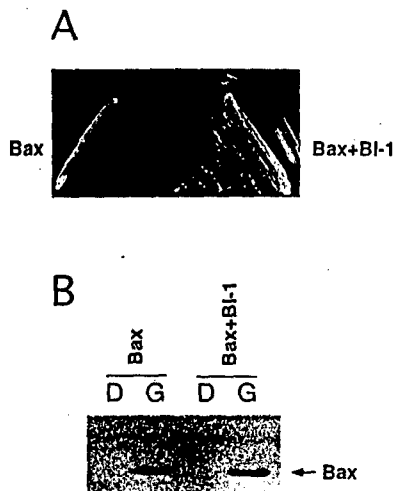


Figure 1. BI-1 Suppresses Bax-Induced Yeast Cell Death

Either control vector or BI-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring YEp51-Bax).

(A) Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4 day incubation at 30°C.

(B) Protein extracts were prepared from QX95001 transformants used in (A) that contained control or BI-1-encoding plasmids. Cells were grown in glucose-containing medium (D = dextrose) and then transferred to galactose-containing medium (G) for 20 hr. Total protein extracts (20 µg/lane) were subjected to SDS-PAGE and immunoblot analysis using anti-mBax antiserum.

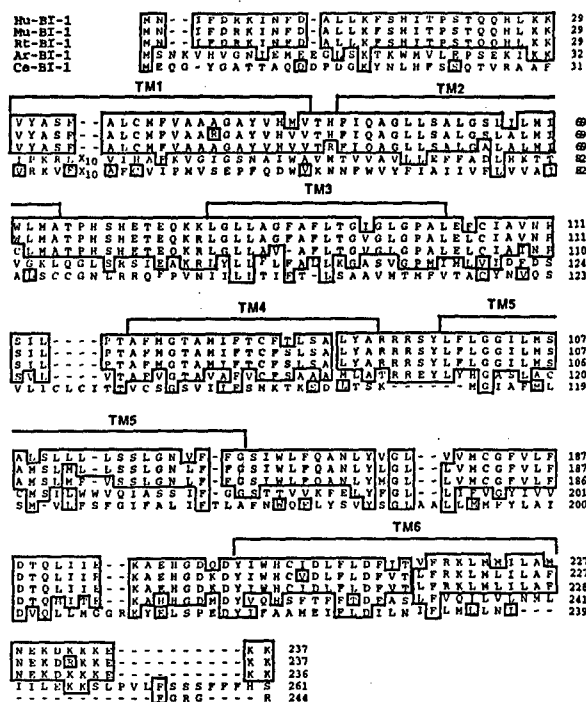


Figure 2. BI-1 Is an Evolutionarily Conserved Protein with Several Predicted Membrane-Spanning Domains

The predicted amino acid sequences of human, rat, and mouse BI-1-proteins, as well as the homologous *C. elegans* and *Arabidopsis* proteins are aligned, with identical residues in boxes. The predicted 6 TM domains of the human BI-1 protein are indicated in brackets.

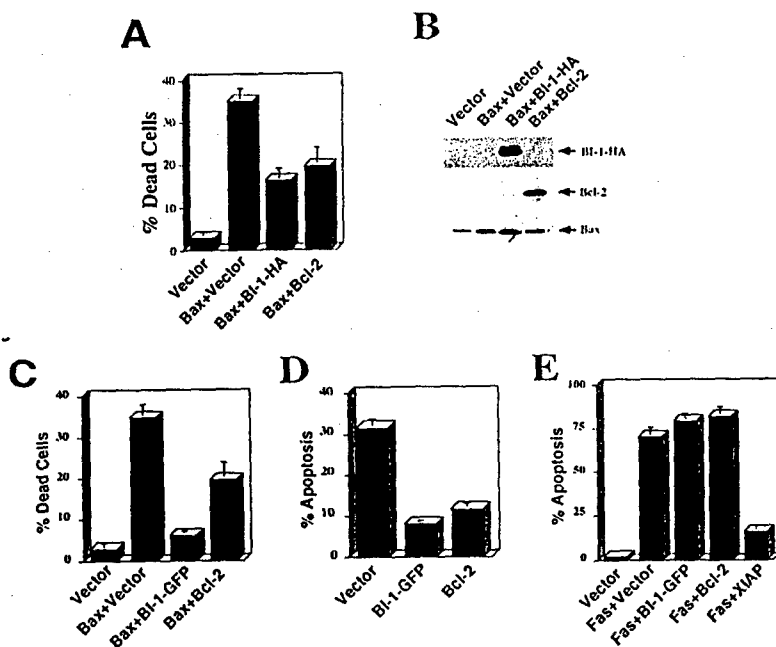


Figure 3. BI-1 Inhibits Cell Death Induced by Bax or Serum Withdrawal but Not Fas

(A) BI-1 protects against Bax-induced apoptosis in 293 cells. 293 cells were transfected with either 9 µg of vector control, or cotransfected with 3 µg of Bax plasmid and 6 µg of either control vector (pcDNA3) or plasmids encoding BI-1 (with a C-terminal HA tag) or Bcl-2. One day after transfection, floating cells and adherent cells (after trypsinization) were pooled. A portion of the pooled cells was subjected to vital dye trypan blue exclusion assay counting at least 300 cells (mean ± SD; n = 3).

(B) Extracts were prepared from another portion of the transiently transfected 293 cells described in (A) above and subjected to SDS-PAGE/immunoblot analysis. The blot was sequentially probed with anti-HA monoclonal antibody, anti-hBax antiserum, and anti-Bcl-2 antiserum, with stripping between each detection. The predicted band corresponding to the ~30 KD BI-1-HA protein is indicated with an arrow.

(C) 293 cells were transiently transfected with either vector control (9 µg) or cotransfected with 3 µg of Bax plasmid and 6 µg of either BI-1-GFP or Bcl-2 plasmid. Trypan blue dye

positive cells (%) were determined 1 day later (mean ± SD; n = 3). The expression of GFP-tagged BI-1 was verified by fluorescence microscopy and by immunoblotting using an anti-GFP monoclonal antibody (now shown).

(D) GM701 cells were cotransfected with a β-gal reporter plasmid (0.5 µg) and the indicated plasmids (4.5 µg each). 18 hr after transfection, cells were washed and cultured in DMEM containing 0.1% FBS for another 30 hr. Floating and adherent cells were fixed and stained with X-gal. The percentage of blue cells (transfected) with apoptotic morphology was determined (mean ± SD; n = 3).

(E) 293 cells were transfected with either vector control (8.5 µg) or cotransfected with 0.5 µg of Fas-encoding plasmid and 8 µg of either vector, or BI-1-GFP-, or Bcl-2- or XIAP-encoding plasmids. GFP-encoding plasmid (0.5 µg) was included in all transfections. 20 hr after transfection, both floating and adherent cells (after trypsinization) were pooled, fixed, and stained with DAPI (Zha et al., 1996). The percentage of GFP-positive cells with fragmented or condensed nuclei (apoptotic) was determined (mean ± SD; n = 3).

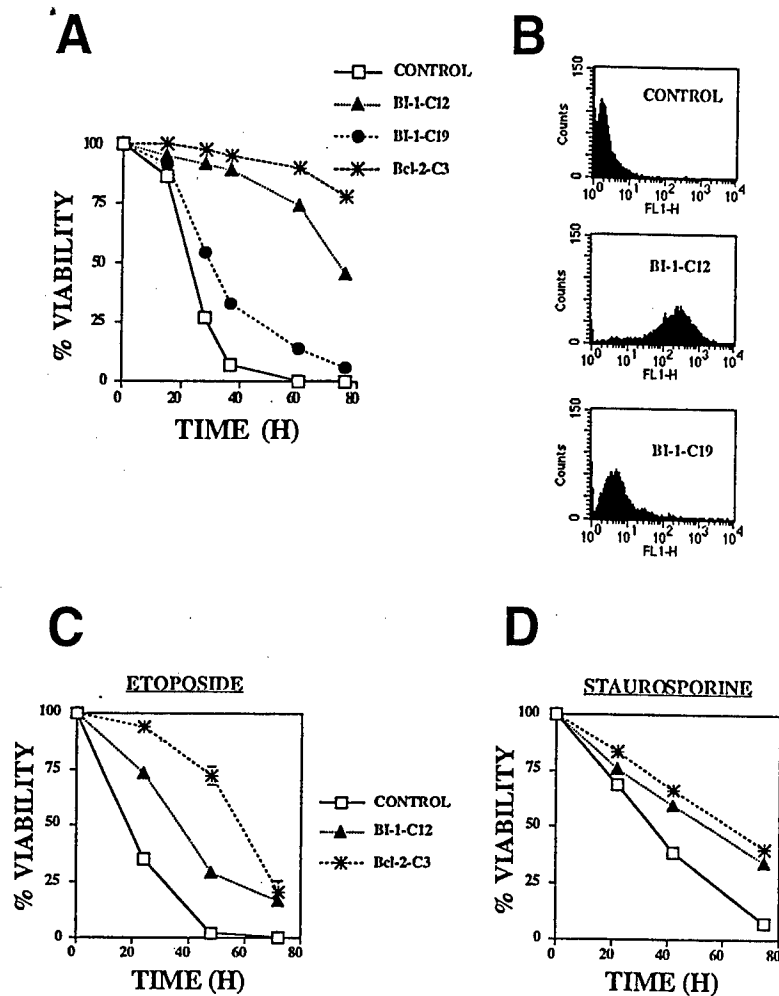


Figure 4. BI-1 Protects against Multiple Apoptotic Stimuli in FL5.12 Lymphocytes

(A) BI-1 inhibits IL-3 withdrawal-induced apoptosis in FL5.12 cells. Stably transfected cell lines expressing GFP-tagged BI-1 or untagged Bcl-2 were generated by electroporation. Independent clones were obtained by limiting-dilution for BI-1 (clone 12 and 19) and Bcl-2 (clone 3). Cells were grown to a density of $\sim 5 \times 10^5$ cells/ml before removing IL-3 from the medium. At various times thereafter, samples were removed and subjected to trypan blue dye exclusion assay (mean $\pm$ SD; $n = 3$).

(B) Expression of GFP-tagged BI-1 assessed by FACS analysis. Stably transfected FL5.12 cells containing either a negative control plasmid (pcDNA3) (top panel) or plasmid encoding BI-1-GFP were analyzed by FACS. The histograms are presented for clone 12 (middle panel), which expresses BI-1-GFP at high levels, as manifested by a single peak of green fluorescence at >2 logs above the negative control (top panel) and for clone 19 (bottom panel), which expresses BI-1-GFP at levels only ~ 0.5 log above background.

(C and D) FL5.12 cells were cultured with IL-3 and either 5 μ g/ml etoposide (C) or 0.5 μ M staurosporine. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion assay (mean $\pm$ SD; $n = 3$). Some SD bars are obscured by symbols.

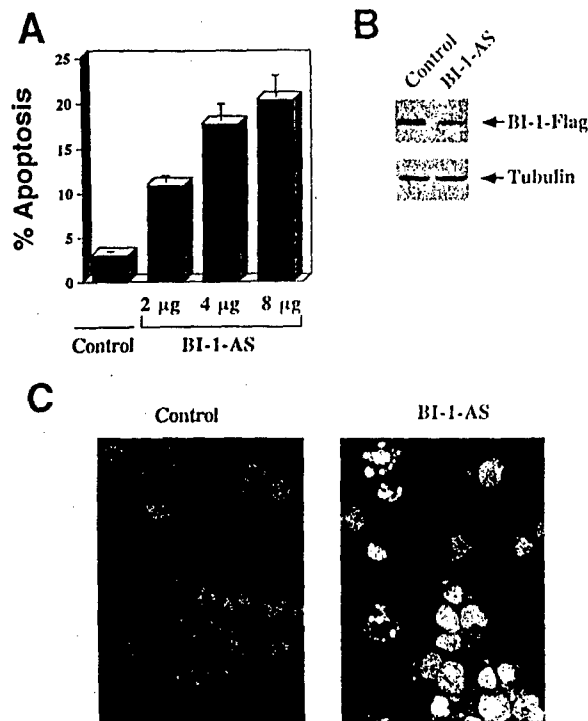


Figure 5. BI-1 Antisense Induces Apoptosis

(A) 293 cells were cotransfected with the indicated plasmids plus 1 μ g of GFP-encoding plasmid. The total amount of plasmid used for each transfection was normalized at 9 μ g. 30 hr after transfection, both the floating and adherent cells were pooled, fixed, and stained with DAPI. The percentage of apoptosis was determined as the ratio of cells with fragmented or condensed nuclei among GFP-positive cells (mean $\pm$ SD; $n = 3$). Data are representative of three independent experiments.

(B) 293 cells were cotransfected with 1 μ g of BI-1-Flag-encoding plasmid in combination with either 8 μ g of pcI-Neo or pcI-Neo-BI-1-AS. 30 hr after transfection, protein extracts were prepared, normalized for total protein content (30 μ g per lane), and subjected to SDS-PAGE/immunoblot analysis using the anti-Flag M2 antibody for detection of BI-1-Flag protein and anti-tubulin antibody to control for loading.

(C) DAPI-stained cells as described in (A) were visualized and photographed under a UV microscope. Note at least four typical apoptotic cells with fragmented or condensed nuclei in the BI-1-AS-transfected population (right panel).

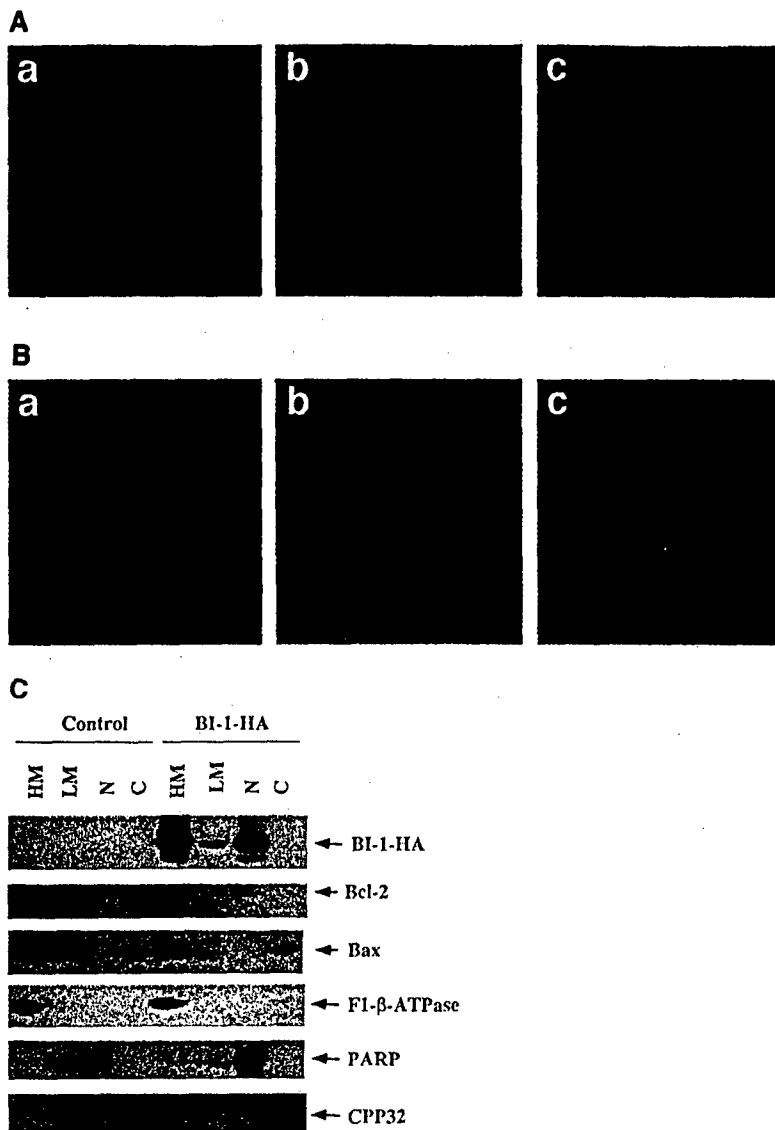


Figure 6. BI-1 Is Localized to Intracellular Membranes and Cofractionates with Bcl-2

(A) Either the parental pEGFP-N2 vector (a) or plasmid encoding BI-1-GFP fusion protein (b) or a plasmid encoding Flag-tagged BI-1 protein (c) was transfected into Cos-7 cells. 18 hr after transfection, cells were seeded in chamber slides for fluorescence microscopy. In (a) and (b), cells were analyzed directly using appropriate filters for visualization of the green fluorescence resulting from GFP. In (c), cells were stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. Cells stained with secondary antibody alone exhibited negligible fluorescence (not shown). Photographs represent ~400 $\times$ original magnification.

(B) BI-1-GFP transfected Cos-7 cells were incubated with the Mitotracker dye before being fixed and visualized by fluorescence confocal microscopy using filters appropriate for the visualization of green (a), red (b), or both (c), resulting from the BI-1-GFP protein and the Mitotracker. Data shown are representative of the majority of doubly stained cells.

(C) 293T cells were transiently transfected with either parental vector (Control) or plasmid encoding HA-tagged BI-1. Cells were lysed 2 days later in a hypotonic solution, and crude subcellular fractionations were prepared. Equivalent proportions of each fraction were subjected to SDS-PAGE/immunoblot analysis using antibodies specific for HA-tag, Bcl-2, Bax, F1 β -ATPase (mitochondria marker), PARP (nuclear marker), and CPP32 (Caspase-3, cytosolic marker).

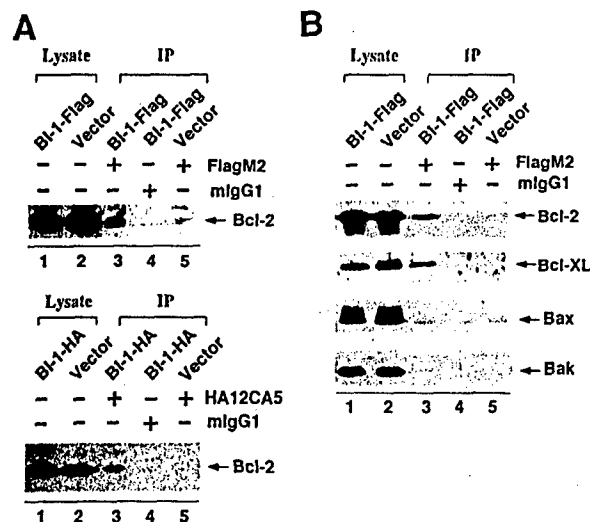


Figure 7. BI-1 Interacts with Bcl-2 in Mammalian Cells

(A) *In vivo* cross-linking. 293 cells were cotransfected with Bcl-2-encoding plasmid and either a control (vector) or plasmids encoding BI-1-Flag (top panel) or BI-1-HA (bottom panel) proteins. 2 days after transfection, cells were washed in PBS and incubated with the membrane-permeable chemical cross-linker DTBP. After cross-linking for 20 min, cells were washed in PBS and lysed in RIPA buffer. Immunoprecipitations were performed using normal mouse IgG1 as a negative control or the anti-Flag M2 (top) or anti-HA 12CA5 (bottom) monoclonal antibodies. Immunocomplexes were reduced (to reverse the cross-linking reaction) and analyzed by SDS-PAGE/immunoblotting using anti-Bcl-2 antiserum. Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2+BI-1 or Bcl-2+vector, respectively (1/20 of the input for lanes 3–5). Lanes 3–5 were loaded with immune complexes precipitated with the indicated antibodies.

(B) Coimmunoprecipitation. 293 cells were transiently transfected with either Bcl-2-, Bcl-X_L-, or Bax-encoding plasmids together with either vector control plasmid or BI-1-Flag-encoding plasmid DNA. 2 days later, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Flag antibody M2 or IgG control. Immune complexes (lanes 3–5) were subjected to SDS-PAGE/immunoblot analysis using antisera specific for Bcl-2, Bcl-X_L, Bax, or Bak. Whole cell lysates (lanes 1 and 2) are from cells transfected with Bcl-2 (top), Bcl-X_L (second), Bax (third), or no plasmid (bottom) together with either BI-1-Flag plasmid (lane 1) or vector control (lane 2), representing 1/40 of the input for immunoprecipitation.

Task #3. Employ classical yeast genetics techniques involving complementation cloning to identify yeast genes that are required for Bax-mediated cytotoxicity.

Bax-resistant strains of yeast were generated and a single strain was identified in which the resistance phenotype segregated as a recessive single-gene defect. Classical complementation cloning methods were then used to identify yeast genes that restored Bax sensitivity, thus identifying subunits of the mitochondrial FoF1-ATPase/proton pump. Targeted gene ablation experiments confirmed that the proton pump is required for Bax-induced killing in yeast. Experiments were then performed with a pharmacological inhibitor of the proton-pump, oligomycin. The addition of oligomycin to cultures of either yeast or human cells resulted in inactivation of the proton pump and resistance to Bax induced cell death. In human epithelial cancer cells, we showed that the proton pump inhibitor also conferred resistance to p53 induced apoptosis, in a cell lines where p53 had been shown to upregulate Bax expression.

These findings thus revealed a functional connection between Bax and the mitochondrial proton-pump. This discovery has important implications for understanding how Bax might function as a channel or pore-like protein. Bax has been reported to translocate from cytosol to mitochondria upon delivery of apoptotic signals to human cells. We and others have shown that Bax induces mitochondrial damage (release of cytochrome c; loss of membrane potential), thus triggering apoptosis and cell death. Our discovery of proton-pump involvement implies either that the proton pump is needed to create a local membrane pH environment that is permissive for Bax channel formation or that the pump is required as a downstream effort of Bax. Experiments are underway to distinguish between these two models.

These findings were published in (6) (reprint attached). A description of the methods employed and the results with figures and legends follows:

Experimental Procedures

Plasmids

YEp51-Bax, pEG202-Bax, and pcDNA-Bax have been described (Zha et al., 1996). The Bax cDNA from pEG202-Bax was subcloned into the EcoRI and XhoI sites of pGilda (gift of C. Kaiser [MIT]). The YCp50 plasmid (ATCC37419) and yeast genomic library (ATCC37415) were obtained from American Type Culture Collection (Rockville, Maryland).

Yeast Methods

Yeast strains and plasmids used for these studies have been described previously (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994; Zha et al., 1996). For generation of Bax-resistant mutant yeast, EGY48 strain was mutagenized with MMNG (Sigma, Inc.) using routine methods (Guthrie and Fink, 1991). After treatment with MMNG for 10 min, yeast cells were grown for 4 hr in YPD media and then transformed by a LiOAc method with the *GAL10* promoter-containing plasmid YEp51-Bax and plated on minimal medium supplemented with required amino acid (MM-A) containing 1% raffinose and 2% galactose. Surviving clones were picked from plates and grown in MM-A with glucose prior to transformation with the *ADH1* promoter-containing plasmid pEG202-Bax (Sato et al., 1994) and selection on MM-A/2% glucose plates. Resistant mutants were mated with Myy290 strain (*Mata*, *his3*, *ura3*, *leu2*), and the resulting diploid cells were subjected to tetrad analysis (Guthrie and Fink, 1991). BRM1 cells displayed precisely 50% inheritance of the Bax-resistant phenotype.

Complementation Cloning

BRM1 cells was transformed with the *GAL1* promoter-containing plasmid pGilda-Bax and grown in MM-A with glucose to a density of $2-4 \times 10^7$ cells/ml. These cells were then transformed using a LiOAc method with 2 μ g of a yeast genomic library in YCp50 (ATCC) and 20 μ g of salmon sperm DNA (transformation efficiency 1×10^4 to 2×10^4 per μ g DNA). Transformed cells were first plated on MM-A with glucose and then replica-plated to MM-A with galactose. From $\sim 2 \times 10^4$ independent colonies tested, 8 clones were identified that did not grow on MM-A/galactose plates. Plasmids were recovered from these 8 candidates, and BRM1 cells were retransformed with these library plasmid together with pGilda-Bax, thus confirming restoration of sensitivity to Bax to approximately the same level as wild-type yeast for 1 of them. The ends of this plasmid were sequenced by primers flanking the cloning site in YCp50 (5'-CGATCATGGCGACCA CACCCGTCCT-3' and 5'-GGTGATGCCGCCACGATGCGTCCG-3'). The DNA sequence results were compared with the Yeast Genomic Data Base using dbFAST (Stanford University).

Yeast Cell Viability Assays

Single colonies of yeast cells transformed with pGilda or pGilda-Bax were grown in 10–20 ml of MM-A/glucose with vigorous aeration at 30°C to an optical density of 0.4–0.5 OD₆₀₀ nm. Cells were pelleted by centrifugation (1000 $\times$ g) for 10 min and washed three times in MM-A/galactose before resuspending in 20 ml of MM-A/galactose and culturing half with 10 μ M oligomycin and half with ethanol solvent control (final 0.1 %). Oligomycin or ethanol was added to MM-A/galactose medium every 12 hr to ensure maintenance of adequate levels of drug. After culturing for various times at 30°C, a 0.5 ml aliquot of cells was removed for trypan blue dye exclusion assay, counting at least 300 total (live and dead) cells. Alternatively, the total cell density of cultures was determined, and either 1000 or 3000 cells were spread on MM-A/glucose plates, followed by culturing at 30°C for 4 days. The number of colonies on plates from the 0 hr cultures was designated as 100%.

Mammalian Cell Transfections and Apoptosis Assays

293T cells were cultured for 12 hr in DMEM-high glucose (4500 mg glucose/l) medium supplemented with 10% fetal bovine serum (FBS) at a density of 10^6 cells in 3.6 ml of medium per 6 cm diameter dish or 3×10^6 cells in 10 ml per 10 cm dish. Fresh medium was exchanged and 4 hr later the cells were transfected with 10 μ g of pcDNA3-Bax versus parental pcDNA3 plasmid (10 cm dishes) or were cotransfected with 1 μ g of pEGFP (Clontech Laboratories, Inc.) and either 9 μ g of pcDNA3-Bax or pcDNA3 control plasmid (6 cm dishes). Four hours after the transfection, the medium was changed with fresh media containing 10 μ M oligomycin, 10 μ M antimycin A, or 0.1% ethanol (solvent). After culturing for an additional 8 hr, both the floating and attached cells were harvested. Half

of the recovered cells were used for immunoblot assays and the remainder were used for either caspase activity assays (Deveraux et al., 1997) or for DAPI staining (Zha et al., 1996).

BRK cells expressing ts-p53 (Subramanian et al., 1995) were maintained in DMEM-high glucose 10% serum medium at the nonpermissive temperature of 39°C and cultured at either 5×10^4 cells/0.1 ml in 96-well flat bottom plates or at 10^6 cells/5 ml in 6 cm dishes for cell death assays. The medium was then changed with fresh 32°C medium, and cells were cultured at 32°C with or without various concentrations of oligomycin or antimycin A for 12 hr. The percentage of viable cells was determined by trypan blue dye exclusion, or cell lysates were prepared for immunoblot analysis of Bax expression.

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 (Zha et al., 1996) or either anti-human Bax or anti-mouse/rat Bax rabbit sera (Krajewski et al., 1994; Krajewski et al., 1995).

ATP Measurements

Cellular ATP content was measured as previously reported (Kane et al., 1985) using firefly lantern extract (Luciferase-luciferin, Sigma) with a luminometer. Data were normalized relative to total protein content of cell lysates.

Measurements of Mitochondrial $\Delta\psi$

Mitochondrial $\Delta\psi$ was measured using DiOC₂ (Molecular Probes, Inc.) as described (Castedo et al., 1996).

Creation of a Mutant Yeast Strain that Displays Resistance to Bax-Induced Cell Death

Yeast strain EGY48 was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) and then transformed with YEp51-Bax, a *LEU2*-marked, high-copy episomal plasmid that produces the mouse Bax protein under the control of the galactose-inducible *GAL10* promoter (Zha et al., 1996). Transformants were plated on leucine-deficient semisolid medium containing galactose, resulting in ~50 viable colonies. To exclude clones that might have survived because of defects in transactivation of the *GAL10* promoter in YEp51-Bax, these transformants were cured of the YEp51-Bax plasmid and then retransformed with the plasmid pEG202-Bax in which Bax is produced from a strong constitutive *ADH1* promoter. The resulting 24 Bax-resistant clones were mated with wild-type Myy290 strain cells, yielding 9 diploids in which sensitivity to Bax-mediated killing was restored, thus suggesting a recessive mutation. Tetrad analysis was then performed for these recessive mutants, with only one (hereafter designated as Bax-resistant mutant-1 [BRM1]) exhibiting 2:2 Mendelian segregation of the Bax-resistance phenotype in a manner consistent with a single gene defect (Figure 1A).

The genomic mutation in BRM1 cells did not interfere with Bax protein production, as determined by immunoblotting (Figure 1B). For these experiments, the wild-type and BRM1 cells were transformed with pGilda-Bax, which produces Bax as a fusion protein with a portion of LexA (used analogous to an epitope tag here), or the pGilda plasmid, which produces only the LexA fragment. The addition of the LexA tag to Bax does not interfere with its intracellular targeting or cell death-inducing function in yeast (Zha et al., 1996).

Complementation Cloning of Yeast Genes that Restore Sensitivity to Bax

The BRM1 cells containing pGilda-Bax were transformed with a centromere-based yeast genomic library. Eight transformants were identified by replica plating that appeared to have a restoration of their sensitivity to Bax-mediated cell death. The plasmids recovered from these eight transformants were then retransformed with pGilda-Bax into BRM1 cells, with only one of these clearly restoring sensitivity to Bax-mediated lethality to wild-type levels (Figure 1A). DNA sequence analysis revealed that this clone contained three genes, including *ATP4*, which encodes subunit 4 of the yeast F_0F_1 -ATPase, a proton pump located in the inner membrane of mitochondria (Weber and Senior, 1997).

ATP4 Is Required for Bax-Induced Lethality in Yeast

Since it has been previously suspected that Bax promotes cell death at least in part through effects on mitochondria (Xiang et al., 1996; Zha et al., 1996), we focused on *ATP4* as a likely candidate gene that is required for Bax-induced killing of yeast. The *ATP4* gene has been previously disrupted in *S. cerevisiae* by *URA3* insertional mutagenesis (Velours et al., 1989; Paul et al., 1992), thus

creating the *ATP4*-deficient strain PVY10. We therefore tested PVY10 cells for resistance to Bax. For these experiments, PVY10 cells were transformed with pGilda-Bax or pGilda control plasmid and grown for various times in galactose-containing medium to induce expression of Bax, and cell viability was monitored by trypan blue dye exclusion. *ATP4*-deficient PVY10 cells were not killed after switching from glucose- to galactose-containing medium (Figure 2A). In contrast, cells of the isogenic wild-type strain (D273-10B/A) that had been transformed with pGilda-Bax began to die within 12 hr after switching to galactose.

To further verify that *ATP4*-deficient PVY10 cells are resistant to Bax-mediated lethality, a clonogenic survival assay was performed in which cells were switched from glucose- to galactose-containing liquid medium for 1 day to induce expression of Bax, and then plated on glucose-based semisolid medium, which suppresses the *GAL1* promoter in pGilda. The plating efficiency of *ATP4*-deficient PVY10 cells was essentially the same, regardless of whether they contained the pGilda-Bax or pGilda plasmids (Figure 2B). In contrast, colony formation by the isogenic wild-type strain was markedly reduced in cells harboring the pGilda-Bax plasmid compared to the control pGilda vector. Clonogenic survival of the wild-type cells began to decline after as little as 6 hr of exposure to galactose, with essentially all cells failing to form viable colonies after a 12 hr exposure. The differences in the kinetics of loss of clonogenic survival and development of trypan blue positivity (compare Figures 2A and 2B) have been observed previously in Bax-expressing yeast (Zha et al., 1996; Jürgensmeier et al., 1997), and likely reflect a commitment to cell death even before loss of plasma membrane integrity. Mating the PVY10 cells with wild-type haploids to create *ATP4*^{+/−} heterozygous diploids restored sensitivity to Bax. Immunoblot analysis demonstrated that the *ATP4* mutation did not prevent production of Bax protein (Figure 2B). We conclude therefore that *ATP4*, which is a nuclear gene that encodes subunit 4 of the yeast mitochondrial F_0F_1 -ATPase proton pump, is required for Bax-mediated killing of yeast.

The Proton-Pump Inhibitor Oligomycin Inhibits Bax-Induced Killing of Yeast

Oligomycin binds to the F_0 portion of the yeast and mammalian F_0F_1 -ATPases and prevents the proton pump from transporting H^+ ions, thus effectively shutting it off (Tzagoloff, 1970). We reasoned that if the proton pump is required for Bax-mediated lethality in yeast, oligomycin should render wild-type yeast resistant to Bax. To test this hypothesis, D273-10B/A strain yeast that had been transformed with either pGilda-Bax or pGilda control plasmids were cultured for various times in galactose-containing medium with or without oligomycin. Oligomycin did not inhibit the growth of yeast under these conditions due to their ability to produce sufficient ATP from anaerobic fermentation (not shown). Oligomycin is non-toxic in yeast and it fails to induce permeability transition pore (PTP) opening in yeast mitochondria (Jung et al., 1997). As shown in Figure 3, oligomycin partially inhibited the Bax-induced killing of yeast, without interfering with production of the LexA-Bax protein. Thus, similar to disruption of the *ATP4* gene, a pharmacological inhibitor of the F_0F_1 -ATPase proton pump suppresses Bax-mediated lethality in yeast.

Respiration Is Not Required for Bax-Induced Killing of Yeast

It was possible that the genetic disruption or pharmacological inhibition of the F_0F_1 -ATPase proton pump indirectly suppressed Bax-mediated lethality in yeast by blocking respiration (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994). To address this question and to further explore the importance of the F_0F_1 -ATPase, we compared the effects of Bax expression on the viability and clonogenic survival of *rho*⁻ yeast as well as on an additional mutant strain of yeast in which the δ subunit of F_0F_1 -ATPase had been inactivated by *URA3* insertional mutagenesis (Giraud and Velours, 1994). Unlike the *ATP4* mutant, both *rho*⁻ and *ATP- δ* -deficient yeast are *petites*. *Rho*⁻ yeast fail to express all proteins encoded in the mitochondrial genome, and thus lack certain proteins that are critical for respiration. Though certain subchains of the F_0F_1 -ATPase proton pump are encoded in the mitochondrial genome, its activity remains partially functional in *rho*⁻ yeast (Schatz, 1968; Giraud and Velours, 1994). In contrast, loss of the nuclear-encoded *ATP- δ* protein results in deficient function of both the F_0 and F_1 components of the proton pump (Giraud and Velours, 1997). These two *petite* strains grew at comparable rates in the absence of Bax (not shown).

The *rho*⁻ yeast transformed with pGilda-Bax began to die when switched to galactose-containing medium, whereas *rho*⁻ cells containing the control pGilda vector did not (Figure 4A). Note however that the Bax-induced cell death and loss of clonogenic survival occurred with delayed kinetics relative to wild-type yeast (compare with Figure 2). Thus, the absence of respiration in *rho*⁻ cells may reduce but does not abrogate Bax-mediated lethality in yeast. In contrast, yeast lacking the δ subunit of the F_0F_1 -ATPase were completely resistant to Bax, despite expressing LexA-Bax protein at levels equivalent to those of the *rho*⁻ cells (Figure 4). We conclude therefore that respiration is not required for Bax-induced killing of yeast, but the F_0F_1 -ATPase proton pump is.

Oligomycin Also Inhibits Bax-Induced Apoptosis and Activation of Cell Death Proteases in Mammalian Cells

No mammalian cells exist that harbor mutations within subunits of the mitochondrial F_0F_1 -ATPase. Thus, to explore whether the proton pump is also required for optimal function of Bax in mammalian cells, one is limited to pharmacological studies employing oligomycin. In mammalian cells, unlike in yeast, oligomycin is toxic and leads secondarily to mitochondrial PTP opening and either apoptosis or necrosis, depending on the particular cells and circumstances evaluated (Castedo et al., 1996). Cell death caused by oligomycin, however, can be delayed by culturing in high glucose-containing medium, which helps to maintain ATP levels via glycolysis (Eguchi et al., 1997; Leist et al., 1997). We therefore explored the effects of oligomycin in human 293T kidney epithelial cells grown in high glucose medium, using a transient Bax transfection assay to induce apoptosis and activation of cell death proteases (caspases) (Zha et al., 1996; Deveraux et al., 1997; Jürgensmeier et al., 1997). For all experiments, oligomycin was added 4 hr after transfections; caspase activity and apoptosis were then measured after an additional 8 hr of culture. Thus, the experiments were performed within the first 12 hr after Bax transfections, before oligomycin caused cell death (>90% trypan blue dye exclusion in control cultures).

As shown in Figure 5A, oligomycin reduced the percentage of apoptotic cells in cultures of Bax-transfected 293T cells in a dose-dependent manner, with concentrations of 1–10 μ M oligomycin preventing approximately half of the Bax-induced cell death. DiOC₆-based measurement of mitochondrial membrane potential demonstrated that these concentrations of oligomycin resulted in hyperpolarization of mitochondria in 293T cells, consistent with a block of the proton pump causing accumulation of H⁺ ions in the intermembrane space of these organelles (Figure 5B). Acute exposure of the cells to the protonophore mCICCP confirmed that the DiOC₆ staining was dependent on the mitochondrial pH gradient (Figure 5B), thus verifying the specificity of this assay.

In contrast to oligomycin, culturing Bax-transfected 293T cells with the respiratory complex III inhibitor antimycin A did not impair Bax-induced apoptosis under these conditions, but did markedly reduce mitochondrial $\Delta\Psi$ (Figure 5B). These findings in mammalian cells thus support the observations obtained with yeast, demonstrating again that respiration is unnecessary for Bax-mediated cell death.

Since gene transfer-mediated overexpression of Bax has been shown to induce activation of caspases that can cleave the substrate peptide DEVD (Deveraux et al., 1997; Jürgensmeier et al., 1997), we measured the effects of oligomycin treatment on Bax-induced activation of DEVD-cleaving caspases using lysates from the transfected 293T cells. As shown in Figure 5C, 293T cells transfected with pcDNA3-Bax contained markedly elevated levels of caspase activity compared to control transfected cells. Addition of 1–10 μ M oligomycin to the cultures substantially reduced the amount of Bax-induced caspase activity (Figure 5C and data not shown).

Under these same conditions, ATP levels were maintained to within ~95% of control levels for 293T cells treated with 1 μ M oligomycin (32 ± 3 nmol/mg protein versus 34 ± 3 nmol/mg protein) and to within ~75% of control levels for cells treated with 10 μ M oligomycin (25 ± 2 nmol/mg protein). Thus, the oligomycin-mediated protection against Bax-induced apoptosis cannot be ascribed to reduced ATP levels. Oligomycin also did not impair production of the expected 21 kDa Bax protein in 293T cells (Figure 5D).

When used at high concentrations in vitro, oligomycin has been reported to inhibit the plasma membrane Na-K ATPase ($IC_{50} \sim 5$ μ M) (Decottignies et al., 1995). We therefore tested the effect of the Na-K ATPase inhibitor ouabain on Bax-induced apoptosis in 293T cells, but found that even at 100 μ M, ouabain had no influence on Bax function (not shown). Based on the above results, therefore, we conclude that the F_0F_1 -ATPase proton pump is either required for optimal function of Bax in 293T cells or enhances Bax's ability to induce apoptosis in these human cells.

Oligomycin Inhibits p53-Induced Apoptosis

The induction of apoptosis in baby rat kidney (BRK) cells by p53 has been shown to be Bax-dependent (Han et al., 1996; Sabbatini et al., 1997). We therefore employed BRK cells that express a temperature-sensitive mutant of p53, and examined the impact of culturing these cells with various concentrations of oligomycin at either the permissive temperature of 32°C where p53 is active and apoptosis ensues or at the nonpermissive temperature of 37–39°C where p53 is inactive. As in the prior experiments, these cells were grown in high glucose medium to maintain ATP levels through glycolysis.

Oligomycin reduced the percentage of apoptotic cells by approximately two-thirds when ts-p53 BRK cells were cultured at 32°C to active p53 (Figure 6A). Under these same conditions, ATP levels were maintained at ~100% of control levels in BRK cells treated with 1 μ M oligomycin and to within ~70% of control for 10 μ M oligomycin (not shown). In contrast, antimycin A had no apparent effect on p53-induced apoptosis in BRK cells (Figure 6A), yet reduced ATP levels more than oligomycin. Immunoblot analysis of BRK cells demonstrated that neither oligomycin nor antimycin A (10 μ M) impaired p53-induced expression of Bax when the cells were cultured at the permissive temperature of 32°C (Figure 6B). Taken together, therefore, these data provide further evidence that Bax-dependent apoptosis requires the mitochondrial F_0F_1 -ATPase proton pump.

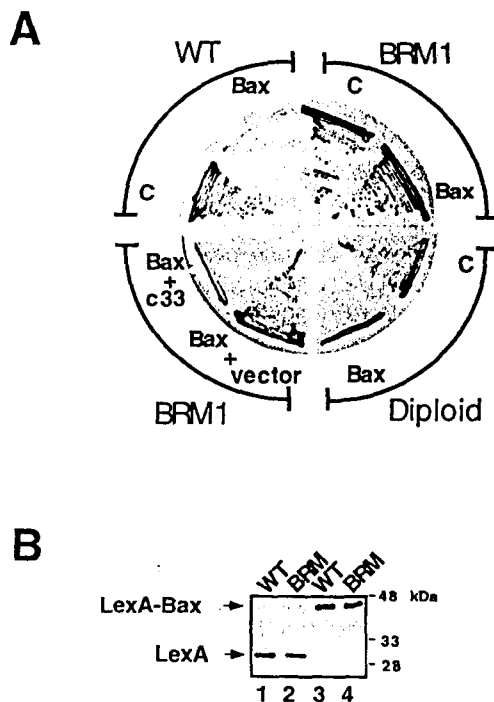


Figure 1. Generation of a Bax-Resistant Yeast Mutant and Identification of Genomic Clones that Restore Bax Sensitivity

(A) Wild-type EGY48 yeast (WT), Bax-resistant-mutant (BRM1), and diploid cells derived from mating Myy290 (wild-type) $\times$ BRM1 were transformed with control plasmid pGilda (C) or galactose-inducible pGilda-Bax (Bax). BRM1 was also cotransformed with pGilda-Bax and clone 33 from yeast genomic library or control vector YCp50. Each transformant was first grown on glucose-based plate and then a colony was restreaked on galactose-containing plates and incubated at 30°C for 4 days.

(B) Immunoblot analysis is shown for lysates (10 μ g) derived from WT yeast (lanes 1 and 3) and BRM1 (lanes 2 and 4) cells transformed with pGilda (produces LexA protein DNA-binding domain without a nuclear localization sequence) (lanes 1 and 2) or pGilda-Bax (produces LexA-Bax fusion protein) (lanes 3 and 4). Antigens were detected using anti-LexA rabbit antiserum.

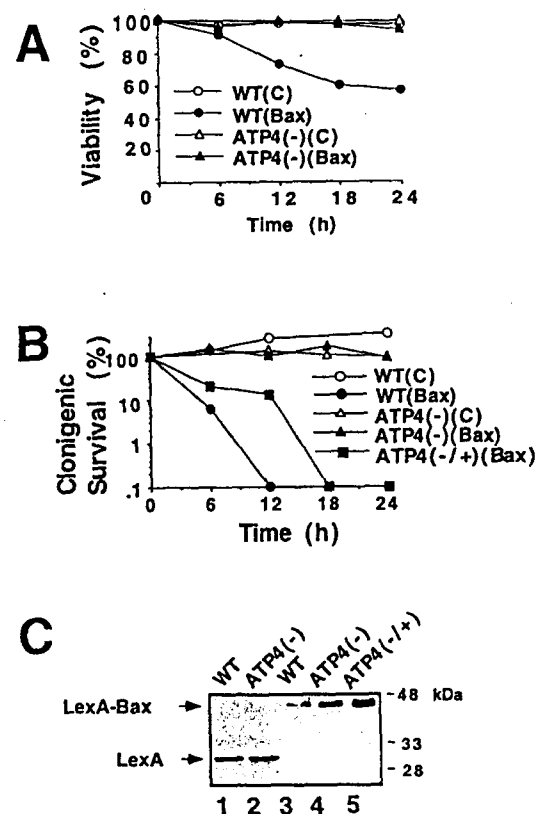


Figure 2. Yeast with Disrupted *ATP4* Gene Are Resistant to Bax-Induced Cell Death

Wild-type D273-10B/A yeast, *ATP4* knockout yeast strain PVY10 (*ATP4*⁻), and heterozygous diploids derived from mating of PVY10 with Myy290 (*ATP4*^{+/+}) were transformed with pGilda (C) or pGilda-Bax. The cells were grown in glucose-based medium and then recultured in galactose-based medium to induce protein expression from the *GAL1* promoter in pGilda plasmids.

(A) The percentage of trypan blue dye excluding cells was determined at various times after switching to galactose-based media (mean $\pm$ SE; $n = 3$; SE bars are obscured by symbols).

(B) Clonogenic survival was determined by recovering cells at various times from galactose-containing medium and plating 1000 cells on glucose-based semisolid medium. Data are representative of at least three experiments.

(C) Immunoblot analysis was performed to assess LexA and LexA-Bax protein levels in cells after 12 hr of culture in galactose-based media, as described for Figure 1.

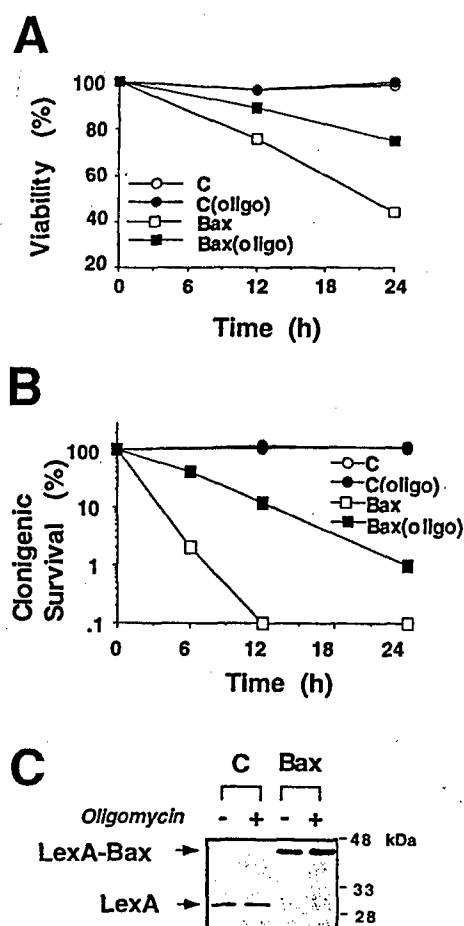


Figure 3. F_1F_0 -ATPase Proton-Pump Inhibitor, Oligomycin, Attenuates Bax-Induced Cell Death in Yeast

Yeast strain D273-10B/A cells transformed with pGilda (C) or pGilda-Bax were initially cultured in glucose-based media, then switched to galactose-containing medium with (closed symbols) or without (open symbols) 10 μ M oligomycin, and the percentage of trypan blue dye excluding cells was determined at various times thereafter (A) or cells were recovered and either 1000 or 3000 cells were plated on glucose-containing semisolid medium (B) (mean $\pm$ SE; $n = 3$; SE symbols are obscured by symbols). In (C), protein lysates (10 μ g) were generated from the same cells after 12 hr of culture in galactose-based medium and analyzed by immunoblotting using anti-LexA antiserum. Lanes 1/2 and lanes 3/4 represent cells containing pGilda or pGilda-Bax, respectively, grown with or without oligomycin as indicated.

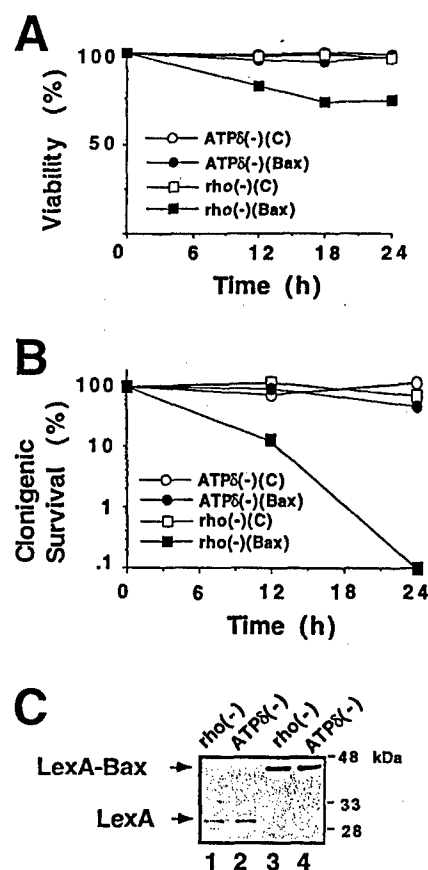


Figure 4. $ATP8$ -Deficient but Not ρ^- petite Yeast Are Bax-Resistant

Strain CGY1 yeast that harbor a $URA3$ -disrupted $ATP8$ gene and ρ^- yeast (both *petite*) were transformed with pGilda (C); open symbols) or pGilda-Bax (closed symbols). Cell viability (A), clonogenic survival (B), and Bax protein levels (C) were measured as described for Figure 2 at various times after switching cells to galactose-containing medium (mean $\pm$ SE; $n = 3$; some SE symbols are obscured by symbols). In (C), lanes 1/2 and lanes 3/4 represent ρ^- and $ATP8$ -deficient yeast transformed with pGilda and pGilda-Bax, respectively.

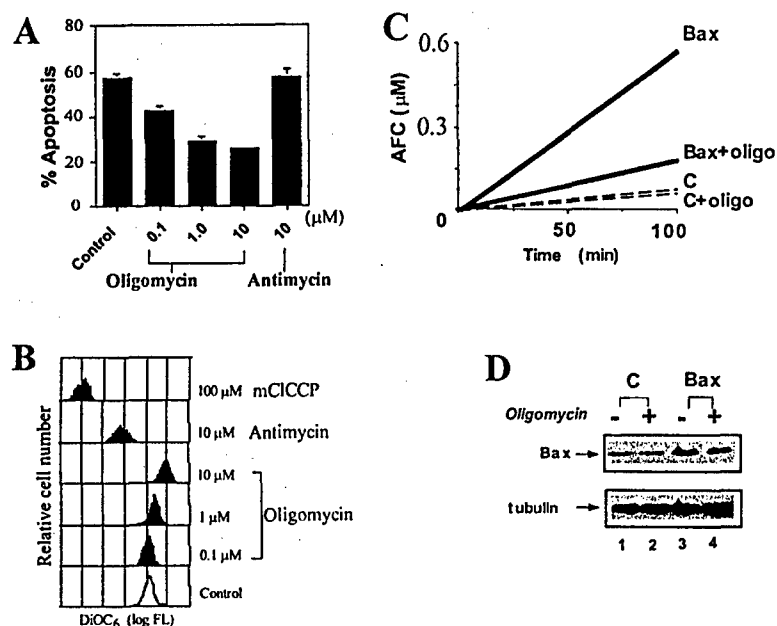


Figure 5. The F_1F_0 -ATPase Inhibitor Oligomycin Suppresses Bax-Induced Apoptosis and Caspase Activation in 293T Cells

(A) 293T cells were cultured in DMEM-high glucose medium to maintain ATP supplies by glycolysis. Four hours after transfection with 9 μ g of pcDNA-Bax (Bax) or control pcDNA (C) plasmids with 1 μ g of pEGFP, the culture medium was exchanged with fresh medium containing or lacking 0, 0.1, 1, or 10 μ M oligomycin or 10 μ M antimycin A. After an additional 8 hr of culture, the cells were collected. In (A), the percentage of GFP⁺ cells with apoptotic morphology was determined by DAPI-staining (mean $\pm$ SD; n = 3).

(B) 293T cells were recovered from cultures of untransfected cells and incubated with DiOC6, followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC6 labeling, an aliquot of the control untreated cells was exposed to the protonophore mClCCCP for 15 min prior to incubation with DiOC6.

(C) Lysates derived from cells that had been cultured with or without 10 μ M of oligomycin

were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1997). Typical substrate hydrolysis progress curves are shown (representative of three experiments).

(D) Aliquots of the same lysates employed for caspase assays were subjected to immunoblot analysis, employing anti-hu Bax antiserum with ECL-based detection. The blot was subsequently reprobed with anti-tubulin antibody to verify loading of equivalent amounts of total protein.

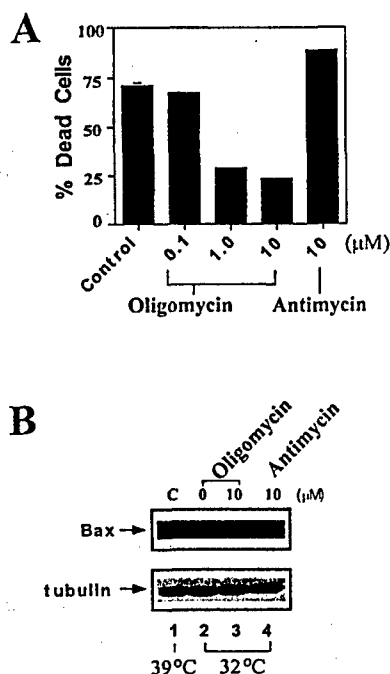


Figure 6. Oligomycin Inhibits p53-Induced Apoptosis

BRK cells that contain ts-p53 (Han et al., 1996) were maintained at a nonpermissive temperature of 39°C, then cultured at 32°C to induce p53 in the presence or absence of 0.1–10 μ M oligomycin or 10 μ M antimycin A. In (A), the percentage of dead cells was determined by DAPI staining 12 hr after shifting to 32°C (mean $\pm$ SD; n = 3). In (B), Lysates were prepared from BRK cells that had been cultured at 39°C (lane 1) as a control (C) or at 32°C (lanes 2–4) without or with 10 μ M oligomycin or 10 μ M antimycin for 12 hr, normalized for total protein content (5 μ g/lane), and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobe with anti-tubulin.

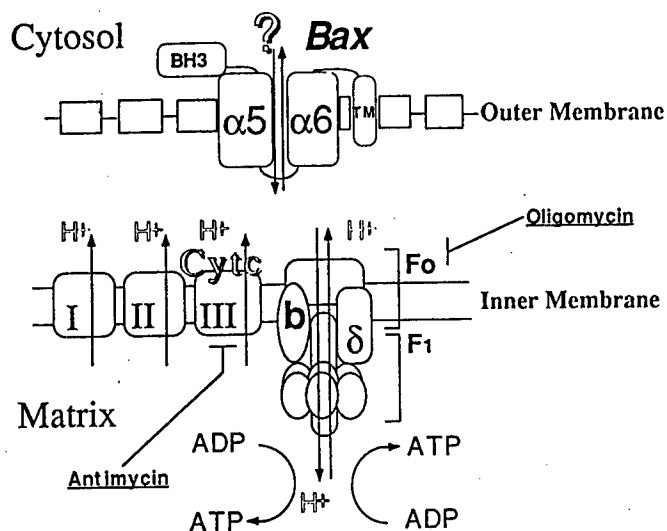


Figure 7. Schematic of Potential Relations between Mitochondrial F₀F₁-ATPase Proton Pump and Bax

The diagram depicts mitochondria, showing the inner and outer membranes. The outer membrane is thought to be porous because of porin. Bax in the outer membrane is anchored via a C-terminal transmembrane (TM) domain. During channel formation, the predicted 5th and 6th α helices of Bax are speculated to penetrate the lipid bilayer. The BH3 domain ($\alpha 2$ helix) is located on the cytosolic side of the membrane and can mediate dimerization with Bcl-2 and related proteins. The F₀F₁-ATPase proton pump resides in the inner membrane. The β (equivalent to subunit 4 in yeast) and δ subunits are indicated. The β and δ subunits of F₀ and F₁, respectively, play important roles in connecting the F₀ proton channel and F₁ ATPase portions together. The transport of H⁺ ions by the proton pump is reversible, and can either consume or generate ATP. Oligomycin shuts off the pump, such that protons cannot be transported in either direction.

The respiratory chain complexes I, II, and III extrude protons into the intermembrane space. Complex III is inhibited by antimycin A and associated with cytochrome c.

Task #4. Derive mutant strains of yeast that are resistant to Bax, and exploit these for functional cloning of human genes that restore Bax-lethality by complementation.

Attempts to exploit Bax-resistant mutant strains of yeast for cloning new Bax-suppressor from humans failed.

Task #5. Characterize the cell death phenotype of Bax in *S. pombe* and begin to explore the potential of employing fission yeast for genetic analysis of the Bcl-2/Bax cell death pathway.

The characterization of Bax induced cell death in the fission yeast *S. pombe* was completed and the results published (7). A reprint is attached. A detailed description of the methods employed and results with figures and legends follows.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. pombe* strain SOP444 (*h⁺ leu1-32 ura4-D18 his7-366 ade6-M210*) was used for all experiments. Cells were maintained in YEL/YES media before transformation and in EMM, lacking the selective amino acids, after transformation (Alfa *et al.*, 1993).

Cloning of Human Bak cDNA

The human *bak* cDNA was cloned by polymerase chain reaction (PCR) amplification from a HepG2 cDNA library using the primers 5'-ATTCCTCGAAACTGGGCTC-3' and 5'-TGGAGTGCACTTGCTAAAG-3'. The resulting PCR product was digested with *Bam*HI and *Hind*III and cloned into the corresponding sites in Bluescript pSK-II (Stratagene, La Jolla, CA).

Plasmid Constructions

The vectors pREP3X (*leu2* marker) and pREP4X (*ura4* marker), containing the thiamine repressible *mtt* promoter, were used for conditional expression of cDNAs in *S. pombe* (Forsburg, 1993). All cDNAs were blunted and subcloned into the *Sma*I site of these vectors. The human *bcl-2* cDNA was excised from pRCMV-Bcl-2 by digestion with *Xba*I and *Hind*III. The human Bax cDNA was obtained from pDNA3-*hubax* by digestion with *Eco*RI. The *bcl-X_L* cDNA was taken from pSKIIBcl-X_L by *Eco*RI digestion. The human *bak* cDNA was excised from pSKIIBak by digestion with *Bam*HI and *Hind*III. The p35 cDNA was excised from pPRM-3K-ORF (Sugimoto *et al.*, 1994) by digestion with *Bam*HI. The *bcl-2* (G145A) mutant was constructed by PCR amplification using the plasmid M1-3 (Yin *et al.*, 1994) as a template and the following primers: 5'-ATCACTCTC-GAGACTATGGCCGACGCTGGGAGA-3' and 5'-ATCGATCTC-GAGTCACTTGTGGCTCAGATAGGC-3'. The resulting PCR product was digested with *Xho*I and subcloned into pREP3X. The proper construction of all plasmids was confirmed by DNA sequencing.

Transformation and Induction of Protein Expression

Transformations were performed using the lithium acetate method (Moreno *et al.*, 1991). Cells were maintained in media (EMM lacking uracil and/or leucine) containing 5 µg/ml thiamine to prevent induction of the *mtt* promoter. Cells were then cultured either on plates or in liquid media in the presence or absence of thiamine. Cells in liquid culture were periodically diluted to sustain log phase growth.

Immunoblot Assays

Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in Laemmli buffer. The resulting lysates were kept at -70°C until used, boiled for 15 min, and briefly centrifuged, and the supernatants were run in 12% polyacrylamide gels. Samples were normalized for cell number, determined by optical density at OD₆₀₀. Immunoblotting was performed using an enhanced chemiluminescence method (Krajewski *et al.*, 1996b). Antibodies used for these studies included polyclonal rabbit antisera huBax 1701, huBak 1764, and huBcl-X 1695 and the anti-human Bcl-2 monoclonal antibody 4D7 (Krajewski *et al.*, 1994-1996a; Reed *et al.*, 1992).

Electron Microscopy (EM)

Cells were harvested at the indicated times by centrifugation, washed once with phosphate-buffered saline, and fixed for 30 min in 4 M phosphate buffer containing 3% glutaraldehyde. After two washing steps with 4 M phosphate buffer, cell pellets were embedded in Epon. Cells were postfixated and counterstained with 0.5% osmium tetroxide and 1% uranyl acetate, cut ultrathin, and placed on grids (Krajewski *et al.*, 1993). Sections were imaged using a Hitachi H-600 electron microscope.

Cysteine Protease Enzyme Assays

Lysates (50 µg total protein) were prepared from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 transformants by glass bead disruption, adjusted to 150 µl total volume in buffer A [20 mM *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, 10% sucrose], and added to a single well of a 96-well plate. Reactions were started by addition of 50 µl of fluorogenic tetrapeptide-aminomethylcoumarin (AMC) substrates to a final concentration of 10 µM. The following substrates were used: DEVD-AMC and YVAD-AMC. Fluorescent AMC product formation was measured at excitation 360 nm, emission 460 nm, using a cytofluor II fluorescent plate reader (Millipore, Bradford, MA). The change in fluorescence was measured over 1 h and expressed as the change in fluorescence per hour. 293 cell lysates were prepared in buffer A from 1.8×10^6 cells on 10-cm dishes 24 h after transfection using standard calcium phosphate methods with either 50 µg pCIneo (Promega, Madison, WI) alone, 50 µg total of pCIneo/pClp35, pClBax/pCIneo, or pClBax/pClp35 at equal molar ratios. Fifty micrograms of extract were used per assay point and determinations were made in duplicate.

p35 Inhibition Assays

Lysates (50 µg total protein) from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 double transformants or pRep transformants as a control were prepared by glass bead disruption or, alternatively, 50 ng of recombinant 6His-p35 protein were used. Yeast cell lysates or recombinant p35 were adjusted to a total volume of 100 µl in buffer A, added to a single well of a 96-well plate, and 1 U of recombinant purified active CPP32 protease (Nicholson *et al.*, 1995) was added in a volume of 75 µl. The substrate DEVD-AMC was then added in 25 µl of buffer A to a final concentration of 10 µM and release of the fluorogenic AMC product was monitored as described above. Inhibition of CPP32 activity by lysates or recombinant purified p35 protein was expressed as a percentage relative to the DEVD-AMC cleaving activity obtained when 1 U of CPP32 is combined with 75 µg of Rep3X control extract.

Mammalian Cell Viability Assay

293 cells (2×10^5 /well) in 6-well plates were transfected by calcium phosphate precipitation with pCIneo/pClBax/pClp35 as appropriate in an equal molar ratio. One-fifth the amount of pRC π V- β -galactosidase expression vector was cotransfected as a marker for transfected cells. Twenty-four hours after transfection, cells were fixed and LacZ-expressing cells were visualized using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside as a substrate. Percentage of death was determined as the ratio of round blue cells to total (round + flat) blue cells $\times$ 100. Experiments were done in triplicate and are expressed as the mean value $\pm$ SD.

RESULTS

Expression of Bax or Bak Confers a Lethal Phenotype in S. pombe

cDNAs encoding the human Bax and Bak proteins were subcloned into the *S. pombe* vector pREP3X, which utilizes an *nmt* ("no message with thiamine") promoter for conditional expression of heterologous cDNAs. The presence of thiamine in the growth media represses expression of this promoter, whereas removal of thiamine induces the promoter with a lag time of ~12 h (Maundrell, 1990). *S. pombe* cells (strain SOP444) were transformed with the Bax and Bak expression plasmids or the parental pREP3X plasmid as a control. Transformants were initially plated on media containing thiamine, thus repressing the *nmt* promoter, and then single colonies were isolated, streaked onto plates containing or lacking thiamine, and assayed for their ability to grow. As shown in Figure 1A, yeast calls carrying the Bax expression plasmid were markedly impaired in their ability to grow on plates lacking thiamine compared with cells containing the control plasmid. In contrast, transformants containing the Bax and control plasmids grew equally well on thiamine-supplemented medium. Similar results were obtained for Bak (our unpublished results).

To determine whether Bax and Bak induce cell death as opposed to a reversible growth arrest, yeast cells that had been transformed with the Bax, Bak, or control plasmids were cultured in liquid media without thiamine to induce the *nmt* promoter. Aliquots of these cultures were then removed at various times and plated onto solid media containing thiamine. If the growth inhibition mediated by Bax and Bak was reversible, then plating the cells on thiamine-containing medium would be expected to rescue the cells and result in colony-forming units at frequencies comparable to cells carrying the control plasmid. Conversely, if Bax and Bak kill *S. pombe*, then the cells should not be rescuable on thiamine-supplemented medium.

As shown in Figure 2, A and B, culturing cells that contained Bax or Bak expression plasmids in thiamine-deficient medium (to induce the *nmt* promoter) resulted in a time-dependent decline in the numbers of viable clonogenic cells that could be subsequently recovered on plates containing thiamine, with >75% inhibition of viable colony formation within ~10 h and nearly complete suppression of colony formation occurring within ~14 h (data normalized relative to efficiency of rescue of cells containing the pREP parental plasmid as a control). Using trypan blue staining, we observed the appearance of dying cells around 10–14 h (our unpublished results), consistent with the plating results. These data are consistent with a lethal effect of Bax and Bak on *S. pombe*.

Bcl-2 and Bcl-X_L Rescue S. pombe from Lethal Effects of Bax and Bak

Bcl-2 and Bcl-X_L are anti-apoptotic members of the Bcl-2 protein family that have been reported to suppress the cell death-promoting effects of Bax and Bak in mammalian cells (Oltvai *et al.*, 1993; Chittenden *et al.*, 1995a,b; Farrow *et al.*, 1995). Bcl-X_L can heterodimerize with both Bax and Bak, whereas Bcl-2 interacts efficiently with Bax but more poorly with Bak (Farrow *et al.*, 1995). Certain loss-of-function mutants of Bcl-2, such as Bcl-2 (G145A), fail to heterodimerize with Bax and also do not suppress apoptosis (Yin *et al.*, 1994). To determine whether the expression of Bcl-2 or Bcl-X_L can rescue fission yeast from Bax- or Bak-induced cell death, *S. pombe* cells containing Bax or Bak expression plasmids were transformed with expression plasmids encoding wild-type Bcl-2, mutant Bcl-2 (G145A), or Bcl-X_L under the control of the *nmt* promoter in pREP4X which carries a URA4 selectable marker as opposed to the LEU2 marker in pREP3X plasmid from which Bax and Bak were expressed.

After initial plating on thiamine-containing medium to repress the *nmt* promoter and thus prevent expression of Bax and Bak, single colonies of these transformants were streaked onto plates that contained or lacked thiamine. Both Bcl-2 and Bcl-X_L restored growth to Bax- and Bak-expressing cells when plated on thiamine-deficient medium, as illustrated by the representative experiment shown in Figure 1B. In contrast, Bax- and Bak-expressing cells that contained the plasmid encoding the mutant Bcl-2 (G145A) protein failed to grow when streaked on thiamine-deficient medium. Comparable growth was observed for all transformants when streaked onto plates containing thiamine-supplemented medium, demonstrating the specificity of these results (Figure 1B). Bcl-X_L consistently afforded more protection than Bcl-2 in these assays, not unlike some results involving mammalian cells (Gottschalk *et al.*, 1994). Similar results were obtained with ~10 other independent clones of each transformation.

The ability of Bcl-X_L and Bcl-2 to rescue Bax- and Bak-expressing *S. pombe* cells was also observed in experiments where cells were transiently cultured in thiamine-deficient medium to induce the *nmf* promoter in the pREP3X and pREP4X plasmids that contained *bax*, *bak*, *bcl-2*, and *bcl-X_L* cDNAs, and then at various times thereafter were replated onto thiamine-containing medium to shut off expression of Bax and Bak. As shown in Figure 2, 25–50% of the cells that had been cotransformed with the pREP4X-Bcl-X_L plasmid and either the pREP3X-Bax (Figure 2C) or the pREP3X-Bak (Figure 2D) plasmid were rescuable when plated on thiamine-containing plates after a period of growth in thiamine-deficient medium. Similar results were obtained with Bcl-2, but the rescue was less effective compared with Bcl-X_L (our unpublished results). In contrast, the cells transformed with the pREP4X parental plasmid (lacking a *bcl-X_L* or *bcl-2* cDNA) and either pREP3X-Bax or pREP3X-Bak were all unrecoverable within 24 h of growth in thiamine-deficient medium, as mentioned above (Figure 2, A and B). Since coexpression of Bcl-X_L allowed cells to survive a transient exposure to Bax or Bak, resulting in increased numbers of viable clonogenic cells, we conclude that Bcl-X_L is capable of abrogating the lethal effects of Bax and Bak in *S. pombe*.

Finally, the effects of Bcl-2 and Bcl-X_L on Bax- and Bak-expressing *S. pombe* cells were examined by growing cells in thiamine-deficient liquid culture medium and spectrophotometrically assessing the cell culture densities over a ~2-day period by measuring the absorbance at 600 nm. As shown in Figure 3, when *S. pombe* cells containing the pREP3X-Bax or pREP3X-Bak plasmids in combination with the pREP4X control plasmid lacking a *bcl-2* or *bcl-X_L* cDNA were switched from thiamine-containing medium to thiamine-free medium, growth as defined by OD_{600 nm} began to cease by ~24 h. In contrast, the cell densities in cultures of *S. pombe* cells that contained the pREP4X-Bcl-X_L or pREP4X-Bcl-2 expression plasmids along with either pREP3X-Bax or pREP3X-Bak continued to increase at 24 h, with faster rates of growth seen in the Bcl-X_L- than the Bcl-2-expressing cells (Figure 3, A and B). Cells that coexpressed the Bcl-2 (G145A) mutant protein with either Bax or Bak, on the other hand, ceased growing after ~24 h, thus verifying the speci-

ficity of the results. Growth of all transformants was comparable in thiamine-supplemented medium (our unpublished results), consistent with the thiamine-mediated repression of the *nmf* promoter.

As an additional control, we examined the effects of Bcl-2 and Bcl-X_L on the growth of *S. pombe* in the absence of Bax or Bak. Cells transformed with the pREP4X-Bcl-2 plasmid and pREP3X, the same parental plasmid from which Bax and Bak were expressed but without the *bax* or *bak* cDNAs, grew at rates comparable to control transformants containing only the pREP4X and pREP3X parental plasmids (Figure 3C). Thus, Bcl-2 by itself did not have an effect on growth. In contrast, yeast cells transformed with pREP4X-Bcl-X_L and pREP3X grew somewhat faster than control transformants containing the pREP4X and pREP3X parental plasmids in some experiments (e.g., Figure 3), but grew at rates essentially identical to control transformants in others. Taken together, these data in Figure 3 support the contention that Bcl-2 and Bcl-X_L specifically suppress the lethal effects of Bax and Bak in *S. pombe*, whereas the Bcl-2 (G145A) mutant does not.

Time Courses of Bak and Bax Protein Accumulation Correlate with Kinetics of Growth Inhibition in S. pombe

The time courses of Bax, Bak, Bcl-X_L, and Bcl-2 protein accumulation were evaluated by immunoblotting of the various *S. pombe* transformants after inducing cells in thiamine-deficient medium. These experiments were performed in parallel with the growth studies described above in Figure 3, thus allowing direct comparisons of expression of these Bcl-2 family proteins with function. As shown in Figure 4, production of the Bax and Bak proteins was either undetectable or just barely detectable by immunoblotting at the earliest time examined (14 h) but became maximal at 24–36 h after seeding cells into thiamine-deficient medium. When compared with the data above in Figure 3, these immunoblotting data therefore suggest that accumulation of the Bax and Bak proteins coincides roughly with the onset of growth inhibition. The observation that 10–15 h of Bax or Bak induction renders cells nonviable (Figure 2) suggests that these proteins can have effects on *S. pombe* cells at modest expression levels.

The time courses of induction of Bcl-2 and Bcl-X_L were similar to those of Bax and Bak, as might be expected given that the same *nmf* promoter was used for driving expression of these anti-apoptotic proteins in *S. pombe*. Note that the wild-type and mutant Bcl-2 (G145A) proteins were produced at comparable levels in yeast, thus excluding insufficient production of the mutant Bcl-2 (G145A) protein as an explanation for its failure to rescue cells from Bax- and Bak-induced death. The levels of Bax and Bak produced in yeast containing the Bcl-X_L or Bcl-2 expression plasmids

were typically comparable to or even greater than those seen in cells that expressed Bax or Bak in the absence of these anti-apoptotic proteins, thus discounting lower levels of Bax and Bak protein as the explanation for the rescue of *S. pombe* by Bcl-X_L and Bcl-2 (our unpublished results). Moreover, the maximum relative levels of Bax and Bak reached in fission yeast cells were comparable to those seen in several mammalian tissues when samples were normalized for total protein content (Krajewski *et al.*, 1996a, and our unpublished results). Thus, it cannot be argued that supraphysiological levels of these cell death-promoting proteins were obtained in *S. pombe*. Analysis of control transfected *S. pombe* cells that contained the same pREP 3X and pREP 4X plasmids without *bax*, *bak*, *bcl-X_L*, or *bcl-2* cDNA inserts ("C") revealed no proteins that could be detected by antibodies directed against the human proteins, thus confirming the specificity of the immunoblotting results (Figure 4).

Morphological Analysis of Bax- and Bak-expressing *S. pombe* by EM

The distinct morphological features that characterize apoptosis in animal cells are best demonstrated by transmission EM (reviewed in Wyllie *et al.*, 1980). We therefore analyzed by EM the morphology of *S. pombe* cells undergoing cell death as a result of expressing Bax or Bak. As shown in Figure 5, striking differences were seen in the morphology of control cells that carried plasmids lacking cDNA inserts (A) and the Bax-expressing *S. pombe* cells (B). Bax-expressing cells uniformly developed massive vacuolization of the cytosol. The cytosol also became electron dense ("cytosolic condensation"), similar to previous descriptions of programmed cell death in plant and animal cells. However, the size distribution of control and dying Bax-expressing cells was approximately the same, and thus Bax expression did not induce the cell shrinkage typical of mammalian cell apoptosis. Also, unlike apoptosis in animal cells, plasma membrane blebbing was not observed, but at higher resolution (our unpublished results) invaginations of the plasma membrane were commonly present beneath the cell wall in Bax- and Bak-expressing cells. Foci of chromatin condensation were present in the nucleus of Bax-expressing cells but not in the control cells. No evidence of nuclear fragmentation or of chromatin margination against the nuclear envelope was obtained by EM

analysis. Similar results were obtained for Bak-expressing *S. pombe* cells (our unpublished results). Despite the presence of focal chromatin condensation in Bax- and Bak-expressing *S. pombe* cells, we were unable to detect the presence of fragmented DNA having the oligonucleosomal pattern typical of apoptotic cells by agarose gel electrophoresis.

To explore whether these morphological changes were specifically associated with the cell death process induced by Bax and Bak versus merely a characteristic of producing Bax or Bak protein in this fission yeast, EM analysis was performed for *S. pombe* cells coexpressing Bcl-X_L with either Bax or Bak. Immunoblot analysis confirmed that the relative levels of the Bax and Bak proteins produced in these Bcl-X_L-expressing cells were as high as those seen in cells expressing Bax or Bak alone without Bcl-X_L (our unpublished results). In fact, the levels of Bax and Bak tended to be approximately twofold to threefold higher in the Bcl-X_L-expressing cells (our unpublished results), possibly because these cells were able to tolerate higher levels of these proapoptotic proteins. EM analysis of these cells revealed that about one-half to three-quarters of the Bcl-X_L-expressing cells retained the morphological features of control cells, whereas the others developed the cytosolic condensation, vacuolization, and focal chromatin condensation that was seen in essentially all of the cells that expressed Bax or Bak alone in the absence of Bcl-X_L (Figure 5C). These morphological data are in general accord with the clonogenic assay (Figure 2) which also demonstrates a partial rescue from Bax/Bak-induced death. The incomplete rescue in these assays is possibly due to clonal differences in the copy number of the episomal plasmid from which Bcl-X_L was expressed.

These morphological features of dying *S. pombe* cells were not unique to Bax and Bak, since overexpression of the protein tyrosine phosphatase pyp1 produced many of the same changes but with a delay of ~1 d relative to Bax and Bak. The pyp1 phosphatase induces a G₂-M arrest followed by cell death (Ottillie *et al.*, 1992). EM analysis of pyp1-expressing cells demonstrated elongation of the cells consistent with a G₂-M arrest (our unpublished results), followed ~1 d later by the massive vacuolization and other changes seen in Bax- and Bak-expressing *S. pombe* cells. Multifocal chromatin condensation, however, was less evident in these pyp1-expressing cells compared with Bax and Bak (our unpublished results). Similar morphological features have been described recently for *S. cerevisiae* overexpressing histone H1 (Miloshev *et al.*, 1994), further suggesting that the morphology produced as a result of ectopic expression of Bax and Bak in *S. pombe* is not unique to these mammalian proapoptotic proteins.

Absence of Detectable ICE/CED-3-like Protease Activity in *S. pombe* Cells Undergoing Bax- and Bak-mediated Cell Death

The induction of apoptosis in mammalian cells is typically accompanied by the activation of proteases of the ICE/CED-3 family (reviewed in Martin and Green, 1995). These proteases cleave their substrates specifically after aspartic acid. To address the question of whether *S. pombe* cells undergoing cell death due to the expression of *bax* contain similar protease activities, lysates were prepared from cells after inducing Bax expression from the *nmt* promoter in pREP3X as well as from control cells containing the same plasmid without Bax. In vitro assays for ICE/CED-3 protease activity were then performed using two different fluorogenic peptide substrates that are known to be effective for one or more ICE/CED-3 family proteases: YVAD-AMC and DEVD-AMC (Nicholson *et al.*, 1995). Neither of these substrates was cleaved by *S. pombe* lysates expressing Bax (Figure 6A). In contrast, Bax overexpression in mammalian 293 cells leads to an increase in DEVD-AMC cleaving activity (Figure 6B).

p35 Does not Rescue Bax-induced Cell Death in *S. pombe*

To further explore the possibility that ICE/CED-3-like proteases are involved in the Bax/Bak-mediated cell death process in *S. pombe*, we constructed an expression plasmid containing a cDNA for the baculovirus p35 gene under the control of the *nmt* promoter. The p35 protein binds to and inhibits the enzymatic activities of all known ICE/CED-3 family proteases (Rabizadeh *et al.*, 1993; Hay *et al.*, 1994; Sugimoto *et al.*, 1994; Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). The p35 protein has also been shown to block apoptosis and programmed cell death in a wide range of animal species or cells derived from them, including nematodes, flies, ants, mice, rats, and humans (Clem and Miller, 1994; Pronk *et al.*, 1996; White *et al.*, 1996). Yeast containing the pREP3X-Bax, pREP3X-Bak, or pREP3X plasmids were transformed with pREP4X-p35 or the same vector without a cDNA insert as a control. Cells were then grown to mid-log phase in thiamine-deficient medium to induce the expression of p35 and Bax and Bak. Cell lysates were prepared and tested for their ability to inhibit the activity of the ICE/CED-3-family protease CPP32 using an in vitro protease assay. These experiments demonstrated that an inhibitory activity consistent with p35 was specifically produced in the cells transformed with the p35 but not in cells transformed with the parental plasmid pRep (lysates normalized for total protein content). As an additional control, recombinant purified p35 protein (2.5 ng) was added to this in vitro protease assay, resulting in ~99% inhibition of CPP32 activity (Figure 6C).

Despite the production of biologically active p35 in yeast, no suppression of the lethal phenotype conferred by Bax and Bak was observed in cells that coexpressed p35 with these proapoptotic proteins (our unpublished results). Similarly, Bax- and Bak-induced lethality were also not impaired in yeast in which p35 expression was driven by a constitutive ADH promoter (our unpublished results). In contrast, in 293 cells both the Bax-induced DEVD-AMC cleaving activity (Figure 6B) and Bax-induced cell death are inhibited by overexpression of p35 (Figure 6D). Taken together, these results suggest that *S. pombe* lack protease activities similar to the ICE/CED-3 family proteases that become activated during apoptosis in animal cells. Furthermore, such proteases are evidently not the effectors of cell death induced by Bax and Bak in fission yeast.

Figure 1. Bax/Bak-induced cell death in *S. pombe* and rescue by Bcl-2 and Bcl-X_L. In A, *S. pombe* cells were transformed with a Bax expression plasmid. Four independent clones were tested on selective media with and without thiamine (1-4). In the absence of thiamine Bax is expressed. Controls were transformed with the parent plasmid lacking a *bax* cDNA insert. In B, individual colonies of *S. pombe* transformants containing pREP3X-Bax or pREP3X-Bak with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or REP4X-Bcl-X_L plasmids were streaked onto plates containing or lacking thiamine and growth was monitored 4 d later.

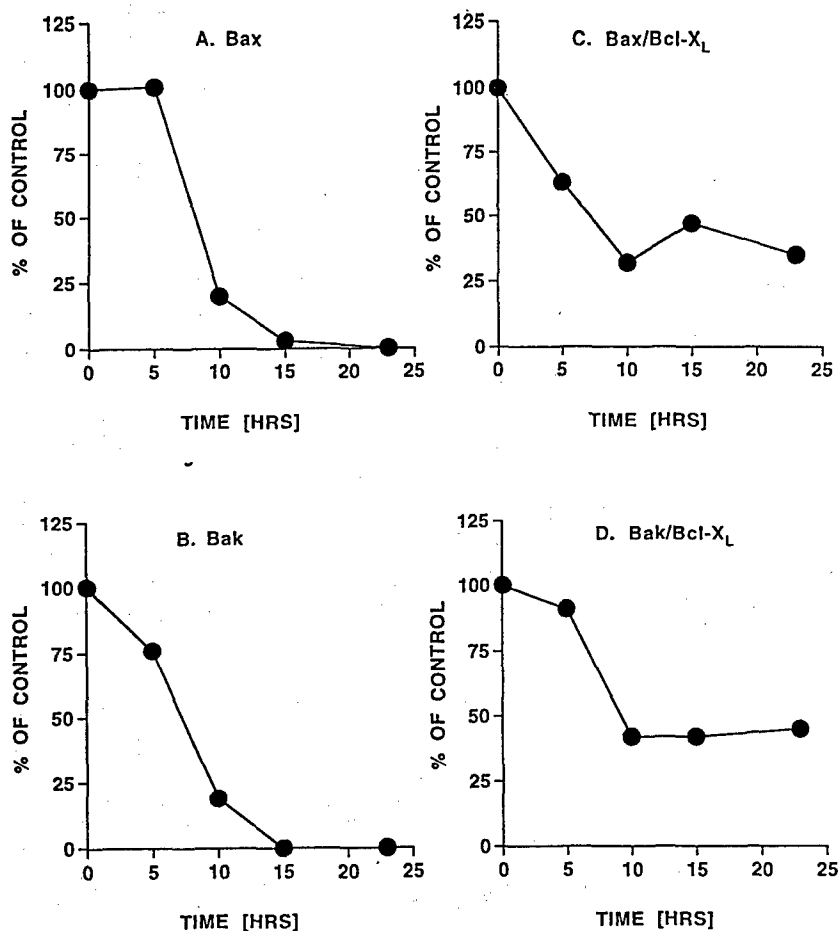
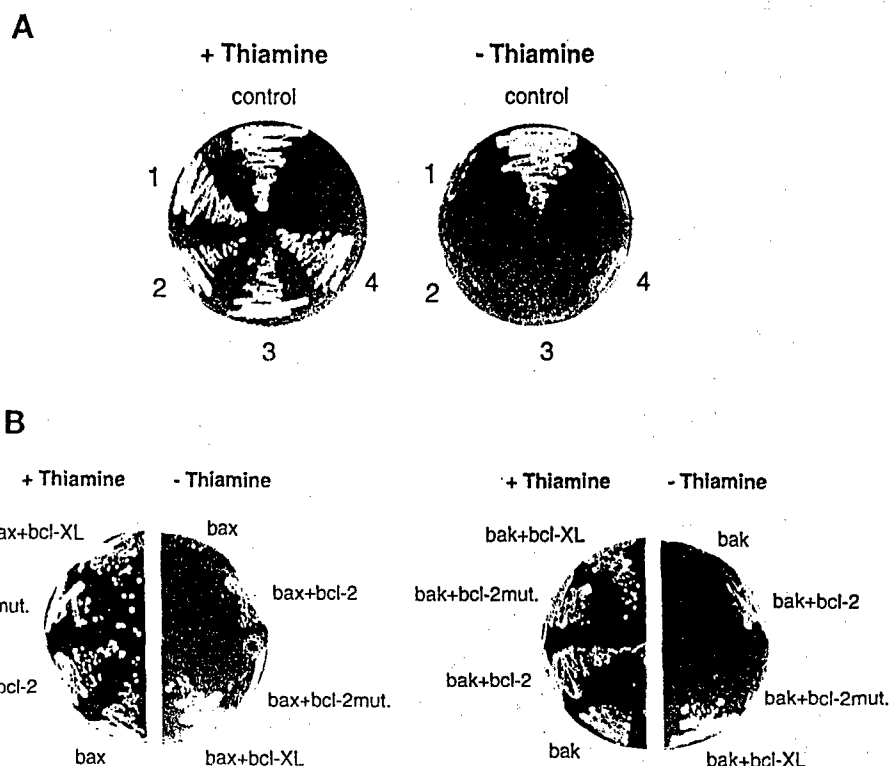


Figure 2. Bax- and Bak-mediated growth inhibition is irreversible. Cells were transformed with pREP3X and pREP4X plasmids either lacking a cDNA insert (parental) or containing *bax*, *bak*, or *bcl-X_L* cDNAs and grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *mtt* promoter. At various times thereafter, cells were plated onto thiamine-containing solid medium, and the relative numbers of colonies were determined based on comparisons with cells cotransformed with the pREP3X and pREP4X parental plasmids. (A) Bax; (B) Bak; (C) Bax and Bcl-X_L; (D) Bak and Bcl-X_L.

Figure 3. Time course of effects of Bax and Bak on growth of *S. pombe*. Cells transformed with pREP3X plasmids containing *bax* or *bak* cDNAs with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or pREP4X-Bcl-X_L plasmids were grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *mtt* promoter. At various times thereafter, relative cell growth was estimated by OD_{600 nm}. Cells were maintained in mid-log phase growth throughout the experiment by dilution in fresh thiamine-deficient medium prior to the OD_{600 nm} reaching 0.7. Data represent calculated theoretical total ODs. C (control) represents cells cotransformed with the pREP3X and pREP4X control plasmids. In A, all transformants contained the pREP3X-Bax plasmid along with various pREP4X plasmids as indicated. In B, all transformants contained the pREP3X-Bak plasmid along with other plasmids. In C, cells cotransformed with pREP3X parental plasmid and either pREP4X parental plasmid (C, ●), pREP4X-Bcl-2 (○), or pREP4X-Bcl-X_L (■). Alternatively, cells were transformed with the pREP4X parental plasmid and either pREP3X-Bax (□) or pREP3X-Bak or pREP3X-Bax (□).

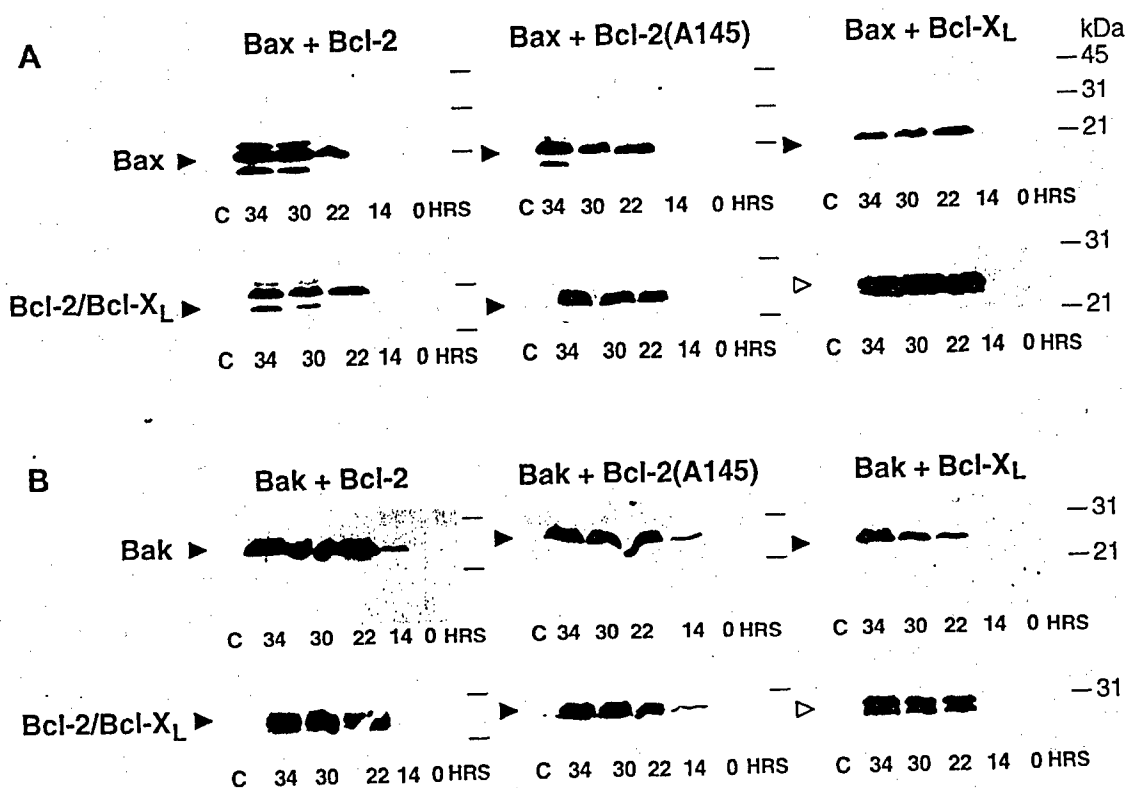
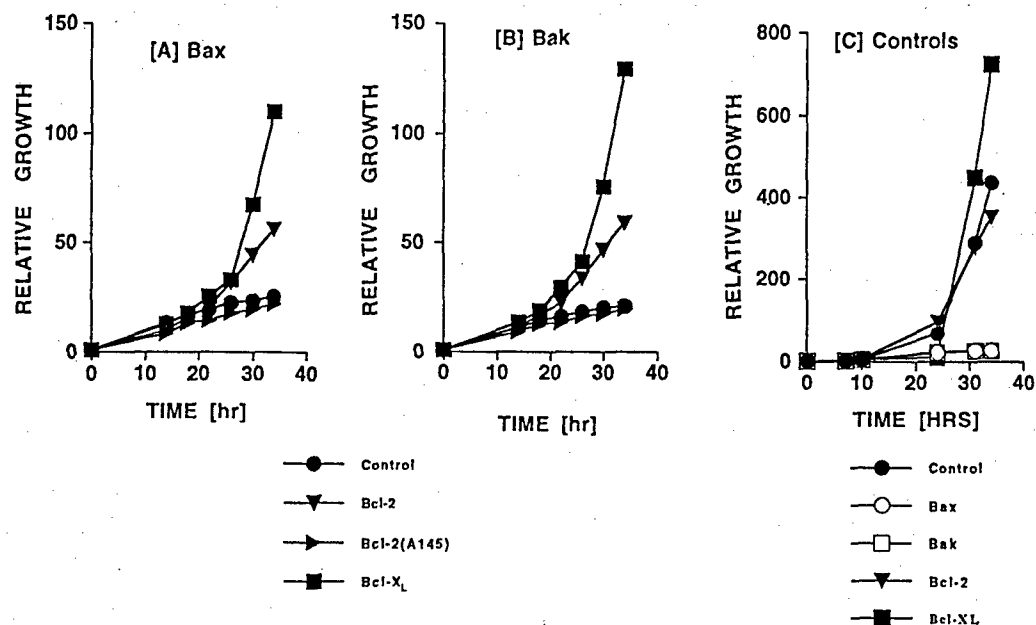


Figure 4. Immunoblot analysis of Bcl-2 family proteins in *S. pombe*. Cells that had been cotransformed with pREP3X plasmids encoding either Bax or Bak and REP4X plasmids encoding Bcl-2, Bcl-2 (G145A), or Bcl-X_L were grown as described in Figure 3, and aliquots of the cells were removed, lysed, and their proteins analyzed by SDS-PAGE/immunoblotting using antisera specific for the human Bax, Bak, Bcl-2, and Bcl-X proteins (Krajewski *et al.*, 1996b). Detection was accomplished with an enhanced chemiluminescence method using a horseradish peroxidase-conjugated secondary goat anti-rabbit antibody as described (Krajewski *et al.*, 1996b). C represents control cells cotransformed with pREP3X and pREP4X parental plasmids. In the rows labeled as Bcl-2/Bcl-X_L, the closed arrows indicate the positions of the Bcl-2 protein, whereas the open arrows indicate the position of the Bcl-X_L protein. The Bcl-X_L protein routinely migrates as a doublet in SDS-PAGE (Krajewski *et al.*, 1994). The smaller band seen in some cases for Bcl-2 probably represents a partial degradation product.

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Figure 5. EM analysis of Bax-expressing *S. pombe*. Cells were transformed with either pREP3X (A), pREP3X-Bax (B), or pREP3X-Bax plus pREP4X-Bcl-X_L (C) and then grown for 1 d in thiamine-deficient medium prior to fixing cells and performing EM analysis.

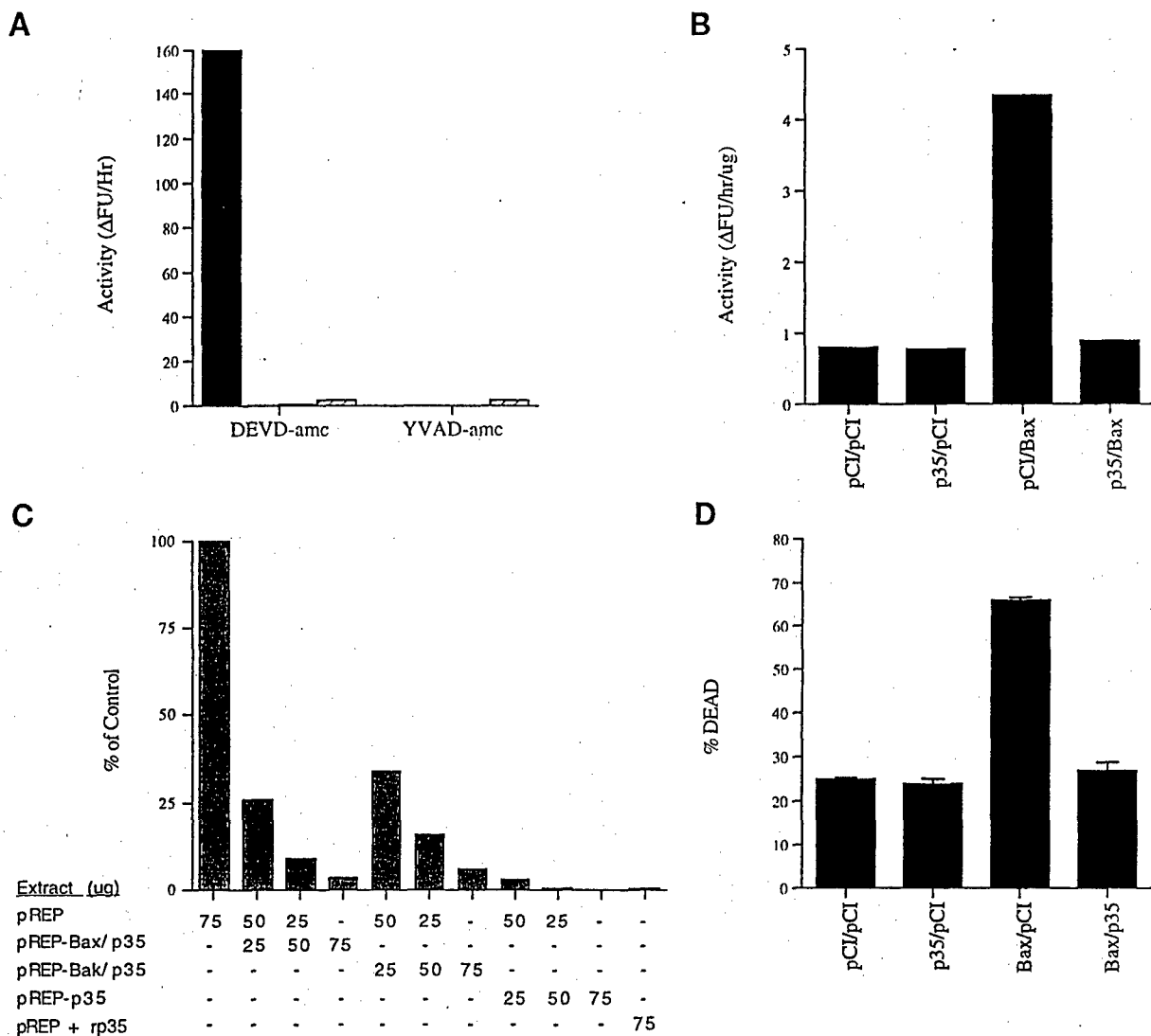


Figure 6. Lack of ICE/CED-3-like protease activity in Bax-transformed *S. pombe* cells. In A, lysates were prepared from *S. pombe* cells cotransformed with pREP4X and either the pREP3X parental plasmid (hatched bar; no activity) or pREP3X-Bax (stippled bar) at 12 h after diluting them into thiamine-deficient medium. The relative amount of release of fluorogenic product (AMC) from the peptide substrates DEVD-AMC and YVAD-AMC in 1 h is shown in arbitrary fluorescence units (Δ FU). As a control, 1 U of purified recombinant CPP32 (solid bar) is shown using DEVD-AMC as a substrate. (This protease cleaves the peptide substrate DEVD-AMC (Nicholson *et al.*, 1995.)) In B, 293 lysates were prepared 21 h after transfection with 50 μ g of pCIneo or 25 μ g each of pCIneo/p35, Bax/pCIneo, or Bax/p35. The relative amount of release of fluorogenic product (AMC) from peptide substrate (DEVD-AMC) (Δ FU) in 1 h is shown in arbitrary fluorescence units. In C, *S. pombe* cells were transformed with either pREP3X-Bax, pREP3X-Bak, or pREP3X parental plasmid, with or without pREP4X-p35 as indicated. Cell lysates were prepared and tested for their ability to inhibit CPP32-mediated cleavage of DEVD-AMC. Specified amounts of each extract were mixed and their ability to inhibit rCPP32 activity was assayed. Results are expressed as percentage of control which corresponds to the activity obtained when rCPP32 is combined with extract from control vector-transformed cells (pREP). The degree of inhibition obtained by addition of 2.5 ng of affinity-purified 6His recombinant p35 (rp35) is included for comparison. In D, 293 cells were transfected as in B except 1 μ g of pRCMV-LacZ *LacZ* expression vector was included with each transfection. Twenty-four hours after transfection, cells were fixed and the presence of functional *LacZ* was detected with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Percentage of dead cells was determined as the ratio of round (dead) blue cells to flat (live) blue cells.

CONCLUSIONS

Using yeast genetics approaches, we discovered a new apoptosis-suppressing gene, BI-1, and we learned that the mitochondrial proton-pump plays an important role in Bax-induced cell death. Our paper on the proton-pump and on BI-1 were the subject of a special minireview in *CELL* (8) about using yeast genetics to study apoptosis, thus reflecting the novelty of the approach and scientific interest in our findings (reprint attached). This new knowledge provides totally unexpected and previously unanticipated starting-points for attempting to restore or replace Bax's cytotoxic function in breast cancers that have lost expression of this important cell death gene.

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APPENDICES

Reprints are provided of all published results.

BIBLIOGRAPHY (Publications resulting from this grant).

1. "Bax Inhibitor-1, a Mammalian Apoptosis Suppressor Identified by Functional Screening in Yeast"

Xu, Q., and Reed, J.C.

Molecular Cell, Vol. 1, 337-346, February, 1998

2. "The Mitochondrial F_0F_1 -ATPase Proton Pump is Required for Function of the Proapoptotic Protein Bax in Yeast and Mammalian Cell"

Matsuyama, S., Xu, Q., Velours, J., and Reed, J.C.

Molecular Cell, Vol. 1, 327-336, February, 1998

3. "Bax- and Bak-induced Cell Death in the Fission Yeast *Schizosaccharomyces pombe*"

Jurgensmeier, J.M., Krajewski, S., Armstrong, R.C., Wilson, G.M., Oltersdorf, T., Fritz, L.C., Reed, J.C., and Otilie, S.

Molecular Biology of the Cell, Vol. 8, 325-339, February, 1997

4. "Death-Defying Yeast Identify Novel Apoptosis Genes"

Shaham, S., Shuman, M.A., and Herskowitz, I.

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Matsuyama, Shigemi

Bax Inhibitor-1, a Mammalian Apoptosis Suppressor Identified by Functional Screening in Yeast

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Summary

The mammalian proapoptotic protein Bax confers a lethal phenotype when expressed in yeast. By exploiting this phenotype, we have identified a novel human Bax inhibitor, BI-1. BI-1 is an evolutionarily conserved integral membrane protein containing multiple membrane-spanning segments and is predominantly localized to intracellular membranes, similar to Bcl-2 family proteins. Moreover, BI-1 can interact with Bcl-2 and Bcl-X_L but not Bax or Bak, as demonstrated by *in vivo* cross-linking and coimmunoprecipitation studies. When overexpressed in mammalian cells, BI-1 suppressed apoptosis induced by Bax, etoposide, staurosporine, and growth factor deprivation, but not by Fas (CD95). Conversely, BI-1 antisense induced apoptosis. BI-1 thus represents a new type of regulator of cell death pathways controlled by Bcl-2 and Bax.

Introduction

Bcl-2 family proteins are centrally involved in control of programmed cell death (PCD), with some inhibiting (Bcl-2 and Bcl-X_L) and others promoting (Bax and Bak) apoptosis (reviewed in Reed, 1994; Yang and Korsmeyer, 1996; Kroemer, 1997). The ability of Bcl-2 family proteins to regulate cell life and death is conserved across evolution. For example, the nematode *Caenorhabditis elegans* contains a Bcl-2 homolog, CED-9, that is essential for the viability of these animals, and expression of the human Bcl-2 protein in *C. elegans* can rescue CED-9-deficient worms (Vaux et al., 1992; Hengartner and Horvitz, 1994). The human Bcl-2 protein can also block apoptotic cell death in insect cells (Alnemri et al., 1992), and human Bcl-2 can protect some mutant yeast strains from death induced by oxidative injury (Kane et al., 1993).

The biochemical mechanism of action of Bcl-2 and its homologs is controversial (reviewed by Reed, 1997a). Recent determination of the three-dimensional structure of Bcl-X_L suggests similarity to the pore-forming domains of some bacterial toxins, particularly diphtheria toxin and the colicins (Muchmore et al., 1996). These toxins function by forming channels in membranes that transport either ions or proteins (Donovan et al., 1981; Cramer et al., 1995). Several members of the Bcl-2 family, including Bcl-2, Bcl-X_L, and Bax, are capable of forming ion-conducting channels in synthetic membranes *in vitro* (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Thus, some Bcl-2 family proteins may have intrinsic activities as channel

proteins, irrespective of other functions related to their ability to interact with several types of proteins in cells (Reed, 1997a).

The mammalian Bax protein confers a lethal phenotype when expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe* (Sato et al., 1994; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). The cell death induced by Bax or its close relative Bak is not attributable to nonspecific toxicity caused by overexpression of a heterologous protein, since antiapoptotic Bcl-2 proteins can rescue yeast from Bax/Bak-induced lethality. Moreover, mutants of Bcl-2 and Bcl-X_L that fail to protect in mammalian cells are inactive at suppressing Bax-induced cell death in yeast. In addition, certain mutations of Bax and Bak that abolish their proapoptotic function in mammalian cells also abrogate their lethal effects in yeast (Zha et al., 1996; Ink et al., 1997). Recently, Bax has been shown to induce release of cytochrome c from mitochondria in yeast (Mannon et al., 1997), which further suggests commonalities in the mechanisms by which Bax induces death in yeast and mammalian cells, given that apoptosis is often associated with release of cytochrome c from mitochondria in mammalian cells (reviewed by Reed, 1997b). In addition, a requirement for the F₀F₁-ATPase proton pump of mitochondria has been demonstrated for optimal cell death-inducing function of Bax in both yeast and mammalian cells (Matsuyama et al., 1998 [this issue of *Molecular Cell*]).

By exploiting the lethal phenotype of Bax in yeast, we reasoned that it should be possible to identify mammalian proteins that suppress Bax function by screening cDNA expression libraries for clones that rescue yeast from Bax-mediated cell death.

Results

cDNA Cloning of Human BI-1

A yeast strain (QX95001) was constructed by transforming the Bax-expression plasmid YEp51-Bax, encoding the full-length mouse Bax protein under the control of the galactose-inducible yeast *GAL10* promoter (Zha et al., 1996), into strain BF264-15Dau (Lew et al., 1991). These yeast cells died upon transfer from glucose to galactose-containing medium, which induces the *GAL10* promoter in this plasmid and leads to accumulation of Bax protein in yeast (Zha et al., 1996). A human HepG2 cDNA expression library (Lew et al., 1991) was transformed into the QX95001 strain and screened for Bax-resistant transformants by plating on galactose-containing solid medium. From a screen of ~10⁸ transformants, 17 displayed Bax resistance. Of these, 4 were determined to be dependent on the introduced cDNA-library plasmid by "con-committant-loss" assay (Ausubel et al., 1991). The nucleotide sequences of 3 of these cDNA clones encoded the same protein, designated

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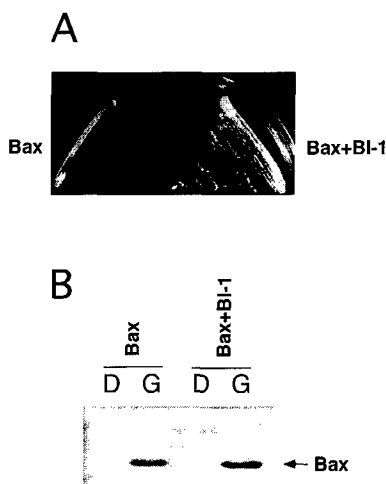


Figure 1. BI-1 Suppresses Bax-Induced Yeast Cell Death

Either control vector or BI-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring YEp51-Bax).

(A) Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4 day incubation at 30°C.

(B) Protein extracts were prepared from QX95001 transformants used in (A) that contained control or BI-1-encoding plasmids. Cells were grown in glucose-containing medium (D = dextrose) and then transferred to galactose-containing medium (G) for 20 hr. Total protein extracts (20 µg/lane) were subjected to SDS-PAGE and immunoblot analysis using anti-mBax antiserum.

BI-1, for Bax Inhibitor-1. BI-1 did not interfere with production of the Bax protein in yeast, as determined by immunoblot analysis (Figure 1).

The Predicted BI-1 Protein Contains Several Membrane-Spanning Segments

All three BI-1 cDNAs obtained by the functional yeast screen contained an open reading frame (ORF) encoding a predicted protein of 237 amino acids. The predicted AUG start codon for this ORF was within a favorable context for translation initiation (Kozak, 1997) and was preceded by an in-frame stop codon. A search against the available nucleotide sequence databases using the BLAST program (Altschul et al., 1990) revealed that BI-1 is essentially identical to TEGT ("testis enhanced gene transcript"), a cDNA previously cloned fortuitously during an attempt to identify testis-specific genes (Walter et al., 1995). The rat TEGT protein has also been described and shares 90% identity (95% similarity) with the human protein (Walter et al., 1994). Additional previously undescribed homologs of BI-1 were also identified by BLAST searches in mouse, and possibly in the nematode *C. elegans* and the plant *Arabidopsis thaliana*. The mouse BI-1 protein, as deduced from ESTs #AA015124, AA275830, AA467259, AA107704, and W59401 is 237 amino acids in length and shares 92% identity (95% similarity) with the human BI-1 protein (Figure 2). The *C. elegans* ORF (EMBL #Q20241) encodes a protein of 241 amino acids that shares 21% overall amino acid identity (37% similarity) with human BI-1. This percentage homology shared by the nematode and mammalian BI-1 is similar to that reported for Ced-9 and Bcl-2,

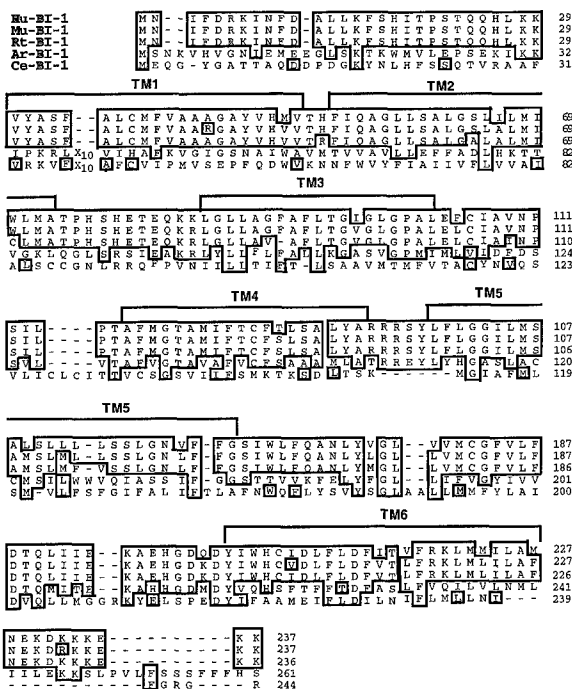


Figure 2. BI-1 Is an Evolutionarily Conserved Protein with Several Predicted Membrane-Spanning Domains

The predicted amino acid sequences of human, rat, and mouse BI-1-proteins, as well as the homologous *C. elegans* and *Arabidopsis* proteins are aligned, with identical residues in boxes. The predicted 6 TM domains of the human BI-1 protein are indicated in brackets.

which share only 23% identity in their amino acid sequences. The putative *Arabidopsis* homolog (EMBL #Z97343) is 261 residues in length, sharing 29% amino acid sequence identity (45% similarity) with the human BI-1 protein.

Based on Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982), the BI-1 protein is predicted to contain 6 or 7 transmembrane (TM) domains. The C termini of the mammalian BI-1 proteins are comprised of basic amino acids resembling some nuclear targeting sequences (Dingwall and Laskey, 1991), but otherwise the predicted proteins lack motifs that suggest a function. Of note, BI-1 does not contain any of the conserved BH domains of Bcl-2 family proteins. By searching a transmembrane protein database (TMbase) using the TMpred program (Hofmann and Stoffel, 1993), the most favored membrane topology for mammalian BI-1 is with 6 membrane-spanning domains and both the N and C termini oriented toward the cytosol. The putative transmembrane segments are predicted to assume mostly an α -helical conformation. Triton X-114 partitioning studies confirmed that BI-1 is an integral membrane protein (data not presented). RNA blot analysis indicated that BI-1 is widely expressed in vivo, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (not shown).

BI-1 Inhibits Bax-Induced Apoptosis in Mammalian Cells

Transient transfection of Bax-encoding expression plasmids induces apoptosis in the human embryonic kidney

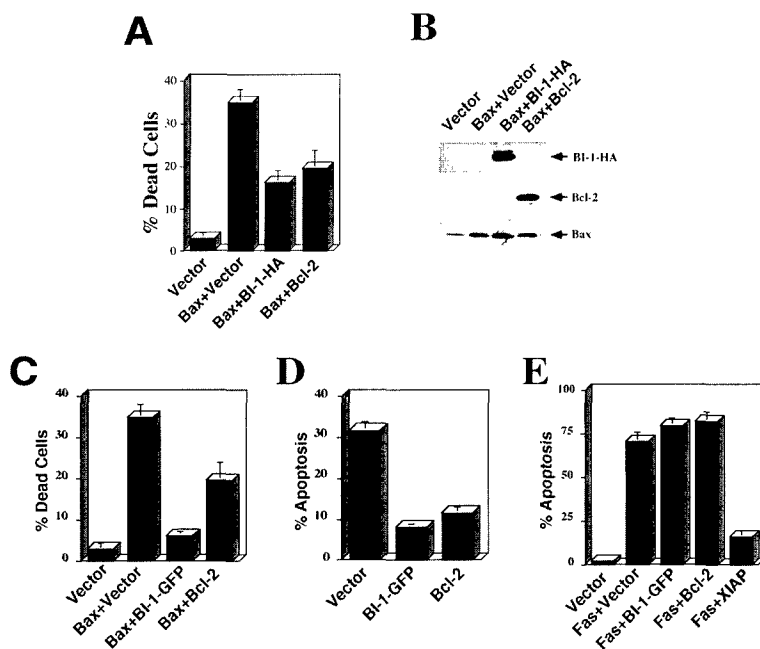


Figure 3. BI-1 Inhibits Cell Death Induced by Bax or Serum Withdrawal but Not Fas

(A) BI-1 protects against Bax-induced apoptosis in 293 cells. 293 cells were transfected with either 9 μ g of vector control, or cotransfected with 3 μ g of Bax plasmid and 6 μ g of either control vector (pcDNA3) or plasmids encoding BI-1 (with a C-terminal HA tag) or Bcl-2. One day after transfection, floating cells and adherent cells (after trypsinization) were pooled. A portion of the pooled cells was subjected to vital dye trypan blue exclusion assay counting at least 300 cells (mean $\pm$ SD; $n = 3$).

(B) Extracts were prepared from another portion of the transiently transfected 293 cells described in (A) above and subjected to SDS-PAGE/immunoblot analysis. The blot was sequentially probed with anti-HA monoclonal antibody, anti-hBax antiserum, and anti-Bcl-2 antiserum, with stripping between each detection. The predicted band corresponding to the ≈ 30 KD BI-1-HA protein is indicated with an arrow.

(C) 293 cells were transiently transfected with either vector control (9 μ g) or cotransfected with 3 μ g of Bax plasmid and 6 μ g of either BI-1-GFP or Bcl-2 plasmid. Trypan blue dye

positive cells (%) were determined 1 day later (mean $\pm$ SD; $n = 3$). The expression of GFP-tagged BI-1 was verified by fluorescence microscopy and by immunoblotting using an anti-GFP monoclonal antibody (now shown).

(D) GM701 cells were cotransfected with a β -gal reporter plasmid (0.5 μ g) and the indicated plasmids (4.5 μ g each). 18 hr after transfection, cells were washed and cultured in DMEM containing 0.1% FBS for another 30 hr. Floating and adherent cells were fixed and stained with X-gal. The percentage of blue cells (transfected) with apoptotic morphology was determined (mean $\pm$ SD; $n = 3$).

(E) 293 cells were transfected with either vector control (8.5 μ g) or cotransfected with 0.5 μ g of Fas-encoding plasmid and 8 μ g of either vector, or BI-1-GFP, or Bcl-2, or XIAP-encoding plasmids. GFP-encoding plasmid (0.5 μ g) was included in all transfections. 20 hr after transfection, both floating and adherent cells (after trypsinization) were pooled, fixed, and stained with DAPI (Zha et al., 1996). The percentage of GFP-positive cells with fragmented or condensed nuclei (apoptotic) was determined (mean $\pm$ SD; $n = 3$).

cell line 293 (Zha et al., 1996). Consequently, pcDNA3-hBax was cotransfected with equal amounts of pcDNA3 parental vector (used as a negative control), pcDNA3-BI-1-HA (HA-tagged BI-1), or pRc/CMV-Bcl-2 (used as a positive control). One day later, both the floating and adherent cells were collected and subjected to the trypan-blue vital dye exclusion assay. BI-1 suppressed Bax-induced cell death in 293 cells to a similar extent as Bcl-2 (Figure 3A). DAPI staining of 293 cell nuclei confirmed that Bax-induced cell death occurred by apoptosis (not shown). Immunoblot analysis demonstrated that BI-1 does not interfere with Bax production in 293 cells (Figure 3B).

BI-1 Inhibits Apoptosis Induced by Growth Factor Deprivation, Etoposide, and Staurosporine, but Not Fas

The effects of BI-1 overexpression on apoptosis induced by a variety of stimuli were explored. For many of these experiments, we employed a green fluorescent protein (GFP)-tagged BI-1, because it provided a convenient marker for transfected cells and because BI-1-GFP accumulated to higher levels than the BI-1-HA protein (not shown). Transient transfection studies in 293 cells confirmed that the BI-1-GFP protein retained biological activity as a suppressor of Bax-induced cell death (Figure 3C).

In the human diploid fibroblast line GM701, $\sim 30\%$ of the cells transfected with control GFP-marker plasmid

developed morphological characteristics typical of apoptosis when deprived of serum for ~ 30 hr. In contrast, GM701 cells transfected with plasmids encoding BI-1-GFP or Bcl-2 were substantially more resistant to serum deprivation, with only $\sim 10\%$ of the cells undergoing apoptosis (Figure 3D). Thus, BI-1 is able to suppress apoptosis induced by growth factor withdrawal in GM701 fibroblasts.

In many types of cells, apoptosis induced by the TNF-family receptor Fas (CD95) is poorly abrogated by Bcl-2 (reviewed by Vaux and Strasser, 1996). We therefore compared the effects of Bcl-2 with BI-1 in 293 cells where apoptosis was induced by transfection of Fas. Cotransfection of either BI-1 or Bcl-2 with a Fas-encoding plasmid failed to prevent apoptosis (Figure 3E). In contrast, Fas-induced cell death was markedly suppressed by cotransfection of a plasmid encoding XIAP, a protein that directly binds to and inhibits caspases required for Fas-induced apoptosis (Deveraux et al., 1997).

To further explore the role of BI-1 as an apoptosis inhibitor, we examined its effects in FL5.12, an interleukin-3-dependent pro-B lymphocyte clone previously shown to undergo apoptosis when deprived of IL-3. FL5.12 cells were stably transfected with plasmids encoding BI-1-GFP or Bcl-2 (as a positive control) and subclones obtained that expressed BI-1 or Bcl-2 at high levels. BI-1 transfectants were uniformly more resistant to apoptosis induction by IL-3 deprivation, with the extent of protection correlating roughly with the levels of

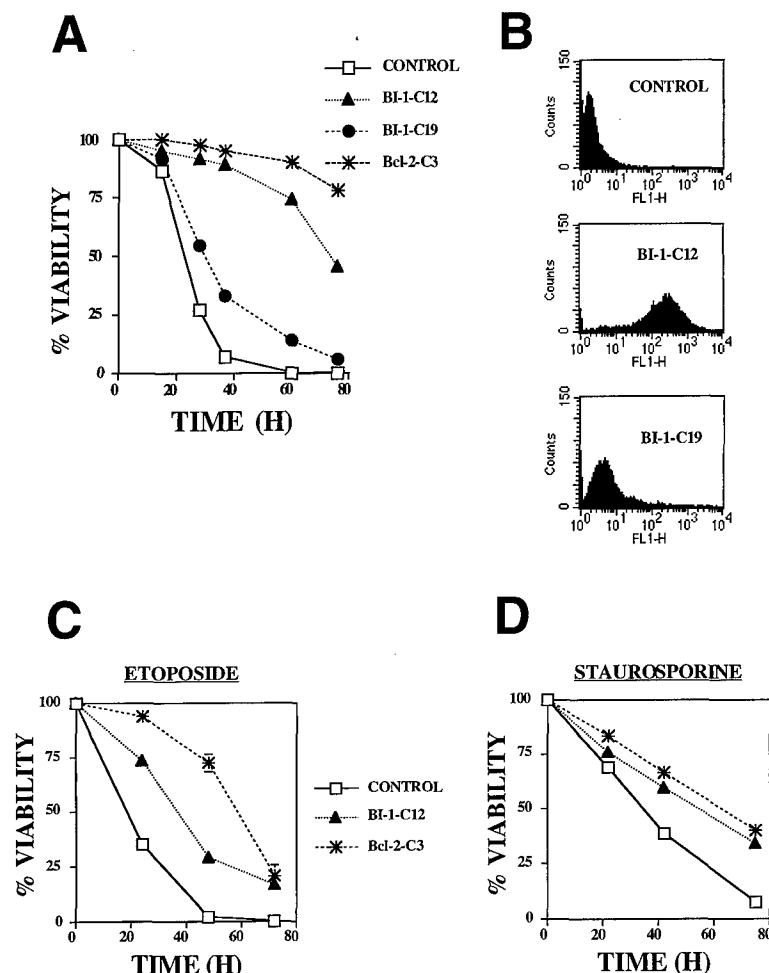


Figure 4. BI-1 Protects against Multiple Apoptotic Stimuli in FL5.12 Lymphocytes

(A) BI-1 inhibits IL-3 withdrawal-induced apoptosis in FL5.12 cells. Stably transfected cell lines expressing GFP-tagged BI-1 or untagged Bcl-2 were generated by electroporation. Independent clones were obtained by limiting-dilution for BI-1 (clone 12 and 19) and Bcl-2 (clone 3). Cells were grown to a density of $\sim 5 \times 10^5$ cells/ml before removing IL-3 from the medium. At various times thereafter, samples were removed and subjected to trypan blue dye exclusion assay (mean $\pm$ SD; $n = 3$).

(B) Expression of GFP-tagged BI-1 assessed by FACS analysis. Stably transfected FL5.12 cells containing either a negative control plasmid (pcDNA3) (top panel) or plasmid encoding BI-1-GFP were analyzed by FACS. The histograms are presented for clone 12 (middle panel), which expresses BI-1-GFP at high levels, as manifested by a single peak of green fluorescence at >2 logs above the negative control (top panel) and for clone 19 (bottom panel), which expresses BI-1-GFP at levels only ~ 0.5 log above background.

(C and D) FL5.12 cells were cultured with IL-3 and either 5 μ g/ml etoposide (C) or 0.5 μ M staurosporine. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion assay (mean $\pm$ SD; $n = 3$). Some SD bars are obscured by symbols.

BI-1-GFP protein. Figure 4A presents results from two clones, one with only modest and another with high levels of BI-1-GFP protein production. Note that even clones with low levels of BI-1-GFP protein exhibited some resistance to IL-3 deprivation, whereas clones expressing high levels of BI-1-GFP protein displayed pronounced resistance to apoptosis induction by IL-3 withdrawal, with the prolongation in cell survival approaching that seen in Bcl-2-transfected FL5.12 cells. BI-1 overexpression also afforded protection in FL5.12 cells against apoptosis induced by etoposide and staurosporine. Comparisons of FL5.12 cell clones with various levels of BI-GFP expression suggested that BI-1 was generally somewhat less effective than Bcl-2 at conferring resistance against these agents (Figures 4C and 4D, and data not shown).

Taken together, the transfection experiments in mammalian cells (Figures 3 and 4) demonstrate that BI-1 has antiapoptotic activity in epithelial, fibroblastic, and hematopoietic cells, and suggest that BI-1 shares functional similarity with Bcl-2 in delaying cell death induced by Bax, growth factor deprivation, staurosporine, and etoposide, but not Fas.

BI-1 Antisense Induces Apoptosis

A BI-1 cDNA was subcloned into pC-Neo in reversed orientation and transiently transfected into 293 cells,

together with a GFP-marker plasmid. The BI-1 antisense (AS) plasmid induced apoptosis of 293 cells in a concentration-dependent manner, whereas control plasmid had no effect (Figure 5A). Lacking antibodies for detection of endogenous BI-1 protein to verify antisense-mediated down-regulation of BI-1 protein, parallel experiments were performed in which 293 cells were cotransfected with plasmids encoding BI-1-Flag protein and the BI-1-AS plasmid. As shown in Figure 5B, the levels of BI-1-Flag protein were markedly decreased in 293 cells that received the BI-1-AS plasmid compared to control transfected cells, as determined by immunoblotting. In contrast, the BI-1-AS plasmid had no effect on the levels of tubulin or other proteins examined, confirming the specificity of the results. These antisense experiments provide further evidence that BI-1 regulates apoptosis in mammalian cells.

BI-1 Is Located in Intracellular Membranes Similar to Bcl-2

The Bcl-2 and Bcl-X_L proteins are associated with intracellular membranes, primarily the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (Krajewski et al., 1993; González-García et al., 1994), while Bax appears to be localized mostly to mitochondria (Zha et al., 1996). To preliminarily explore the

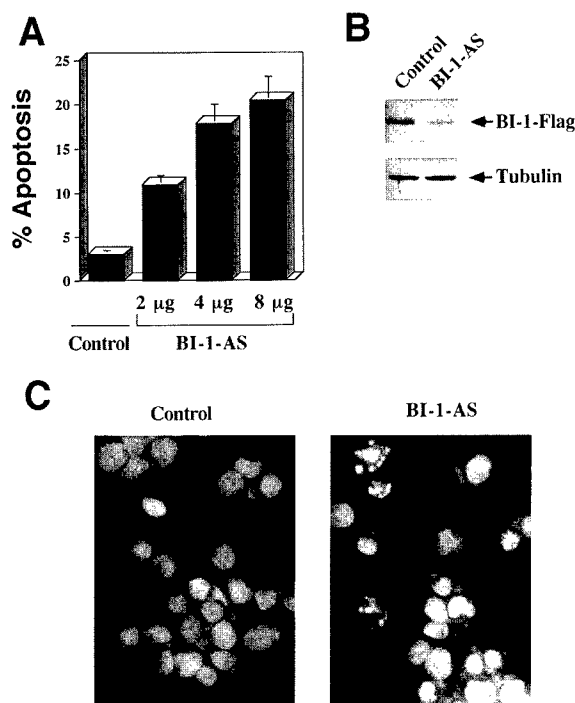


Figure 5. BI-1 Antisense Induces Apoptosis

(A) 293 cells were cotransfected with the indicated plasmids plus 1 µg of GFP-encoding plasmid. The total amount of plasmid used for each transfection was normalized at 9 µg. 30 hr after transfection, both the floating and adherent cells were pooled, fixed, and stained with DAPI. The percentage of apoptosis was determined as the ratio of cells with fragmented or condensed nuclei among GFP-positive cells (mean $\pm$ SD; $n = 3$). Data are representative of three independent experiments.

(B) 293 cells were cotransfected with 1 µg of BI-1-Flag-encoding plasmid in combination with either 8 µg of pCL-Neo or pCL-Neo-BI-1-AS. 30 hr after transfection, protein extracts were prepared, normalized for total protein content (30 µg per lane), and subjected to SDS-PAGE/immunoblot analysis using the anti-Flag M2 antibody for detection of BI-1-Flag protein and anti-tubulin antibody to control for loading.

(C) DAPI-stained cells as described in (A) were visualized and photographed under a UV microscope. Note at least four typical apoptotic cells with fragmented or condensed nuclei in the BI-1-AS-transfected population (right panel).

intracellular locations of the BI-1 protein, GFP-BI-1 protein was expressed in several different adherent cell lines (293, Cos-7, GM701). In all three cell lines, fluorescence microscopy demonstrated that BI-1 is exclusively cytosolic and appears to be associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (Figure 6A and data not shown). In contrast, GFP control protein was diffusely distributed throughout the cells. Only a small portion of BI-1 appears to be associated with mitochondrial membranes, based on two-color analysis using a mitochondria-specific fluorescent dye (Figure 6B). Similar results were obtained using a Flag-tagged BI-1 protein instead of GFP-BI-1 (Figure 6Ac).

The intracellular location of BI-1 was also explored by subcellular fractionation experiments. For this purpose, 293T cells were transiently transfected with the BI-1-HA-encoding plasmid or vector control. Cells were lysed

in hypotonic buffer 2 days later and separated into crude subcellular fractions of nuclei (N), heavy membranes (HM), light membranes (LM), and cytosol (C) by differential centrifugation as described (Wang et al., 1996). As shown in Figure 6C, BI-1 was found mostly in the HM and N fractions (inferred as nuclear envelope based on the GFP fusion localization studies), as determined by immunoblot analysis where the fractions were normalized for cell equivalents. A small proportion of the BI-1-HA protein was also found in the LM fraction. The HM fraction contains mitochondria, lysosomes, and rough ER, whereas the LM fraction contains smooth ER, endosomes, and plasma membranes. As a control for the fractionation procedure, the same blot was reprobed with antibodies specific for the mitochondrial inner membrane protein F₁-β-ATPase, the nuclear protein PARP, and the cytosolic protein CPP32 (caspase-3). Bcl-2 was found essentially in the same subcellular compartments as BI-1, with most of this protein associated with the HM and N fractions (Figure 6C). We conclude therefore that BI-1 is associated with intracellular membranes, based on GFP-tagging experiments, immunofluorescence microscopy, and subcellular fractionation studies.

Recently, it was reported that a subpopulation of Bax molecules in cells are not integrated into membranes, but rather are found within a soluble cytosolic fraction, with the relative proportion of membrane-associated Bax increasing after application of apoptotic stimuli (Hsu et al., 1997). Comparisons of BI-1- and control-transfected 293T cells, however, demonstrated that BI-1 does not substantially change the relative amounts of Bax protein associated with various subcellular compartments (Figure 6C).

BI-1 Associates with Bcl-2 In Vivo

The subcellular fractionation data suggest that BI-1 and Bcl-2 colocalize to the same intracellular membranes. To address the question of whether BI-1 and Bcl-2 physically associate in membranes, we performed *in vivo* cross-linking experiments. Plasmids encoding either Flag-tagged or HA-tagged BI-1 were cotransfected with Bcl-2 into 293 cells. Cells were then incubated 2 days later with the thiol-cleavable chemical cross-linker DTBP. As shown in Figure 7A, both the HA- and Flag-tagged BI-1 proteins could be cross-linked to Bcl-2, suggesting that BI-1 and Bcl-2 come within close proximity to each other in membranes. In contrast, Bax was not cross-linked to BI-1 (data not shown).

To further explore the interaction of BI-1 with Bcl-2 family proteins, we attempted to coimmunoprecipitate BI-1 with Bcl-2, Bcl-X_L, Bax, and Bak. For these experiments, 293 cells, which contain high levels of Bak but very little endogenous Bcl-2, Bcl-X_L, or Bax, were transfected with Bcl-2, Bcl-X_L, or Bax-expression plasmids and either Flag-tagged BI-1 plasmid or empty vector. As shown in Figure 7B, Flag-tagged BI-1 protein specifically coimmunoprecipitated with Bcl-2, and Bcl-X_L but not Bax or Bak. Testing of several deletion mutants of Bcl-2 revealed that the BH4 domain is required for interactions with BI-1 (not shown). This domain is uniquely found in antiapoptotic but not most proapoptotic members of the Bcl-2 family (Reed, 1997a), presumably explaining

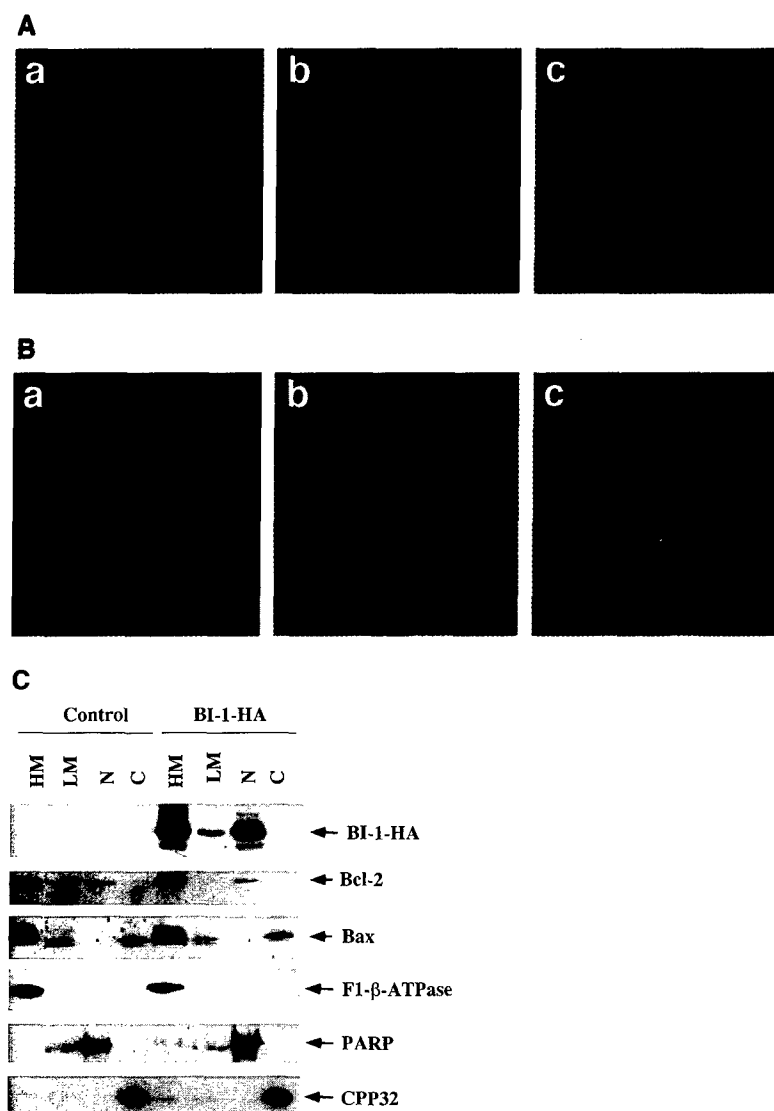


Figure 6. BI-1 Is Localized to Intracellular Membranes and Cofractionates with Bcl-2

(A) Either the parental pEGFP-N2 vector (a) or plasmid encoding BI-1-GFP fusion protein (b) or a plasmid encoding Flag-tagged BI-1 protein (c) was transfected into Cos-7 cells. 18 hr after transfection, cells were seeded in chamber slides for fluorescence microscopy. In (a) and (b), cells were analyzed directly using appropriate filters for visualization of the green fluorescence resulting from GFP. In (c), cells were stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. Cells stained with secondary antibody alone exhibited negligible fluorescence (not shown). Photographs represent $\sim 400\times$ original magnification.

(B) BI-1-GFP transfected Cos-7 cells were incubated with the Mitotracker dye before being fixed and visualized by fluorescence confocal microscopy using filters appropriate for the visualization of green (a), red (b), or both (c), resulting from the BI-1-GFP protein and the Mitotracker. Data shown are representative of the majority of doubly stained cells.

(C) 293T cells were transiently transfected with either parental vector (Control) or plasmid encoding HA-tagged BI-1. Cells were lysed 2 days later in a hypotonic solution, and crude subcellular fractionations were prepared. Equivalent proportions of each fraction were subjected to SDS-PAGE/immunoblot analysis using antibodies specific for HA-tag, Bcl-2, Bax, F1 β -ATPase (mitochondria marker), PARP (nuclear marker), and CPP32 (Caspase-3, cytosolic marker).

why Bcl-2 and Bcl-X_L but not Bax and Bak form complexes with BI-1.

Discussion

By undertaking a functional screen for Bax suppressors in yeast, we have identified a novel human apoptosis inhibitor, BI-1. BI-1 is highly conserved throughout evolution. It shares no identifiable similarity to Bcl-2 family proteins or any other proteins implicated in PCD. Thus, BI-1 represents a novel type of apoptosis modulator. Interestingly, however, the predicted transmembrane topology and intracellular location of the BI-1 protein are somewhat similar to the presenilins, PS-1 and PS-2, which have been implicated in apoptosis and neurodegenerative disease, though no clear sequence homology is shared between BI-1 and these proteins (Wolozin et al., 1996; Guo et al., 1997).

Because BI-1 was identified by its ability to suppress Bax-induced yeast cell death, in theory, it could function upstream of, at the same level as, or downstream of

Bax. For instance, BI-1 could act upstream of Bax, affecting the production of Bax or the targeting of Bax to its proper intracellular locations. Our data, however, suggest that BI-1 has no significant impact on the levels of Bax or its intracellular location. BI-1 does not appear to be associated with Bax, implying that it does not inhibit Bax directly. However, physical interaction is not necessarily required for mutual antagonism among anti- and proapoptotic Bcl-2 family proteins (Simonian et al., 1996; Tao et al., 1997; Zha and Reed, 1997). Given that the BI-1 associates with Bcl-2 and Bcl-X_L in mammalian cells, it is also formally possible that BI-1 functions through a Bcl-2 homolog in yeast. We do not favor this hypothesis, mainly because no Bcl-2 homologs are identifiable in *S. cerevisiae* on the basis of amino acid sequences deduced from the complete yeast genome.

BI-1 might function downstream of Bax, based on the apparent scarcity of BI-1 in mitochondrial membranes. In this regard, our preliminary attempts to localize BI-1 indicate that it is predominantly nonmitochondrial and instead may be mostly localized to the ER. In contrast,

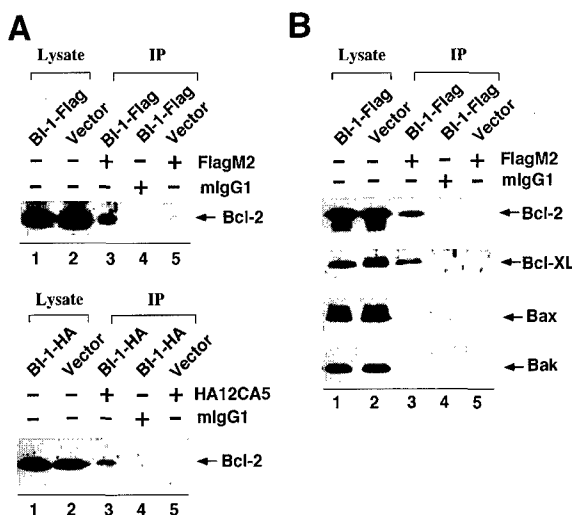


Figure 7. BI-1 Interacts with Bcl-2 in Mammalian Cells

(A) *In vivo* cross-linking. 293 cells were cotransfected with Bcl-2-encoding plasmid and either a control (vector) or plasmids encoding BI-1-Flag (top panel) or BI-1-HA (bottom panel) proteins. 2 days after transfection, cells were washed in PBS and incubated with the membrane-permeable chemical cross-linker DTBP. After cross-linking for 20 min, cells were washed in PBS and lysed in RIPA buffer. Immunoprecipitations were performed using normal mouse IgG1 as a negative control or the anti-Flag M2 (top) or anti-HA 12CA5 (bottom) monoclonal antibodies. Immunocomplexes were reduced (to reverse the cross-linking reaction) and analyzed by SDS-PAGE/immunoblotting using anti-Bcl-2 antiserum. Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2+BI-1 or Bcl-2+vector, respectively (1/20 of the input for lanes 3–5). Lanes 3–5 were loaded with immune complexes precipitated with the indicated antibodies.

(B) Coimmunoprecipitation. 293 cells were transiently transfected with either Bcl-2-, Bcl-X_L-, or Bax-encoding plasmids together with either vector control plasmid or BI-1-Flag-encoding plasmid DNA. 2 days later, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Flag antibody M2 or IgG control. Immune complexes (lanes 3–5) were subjected to SDS-PAGE/immunoblot analysis using antisera specific for Bcl-2, Bcl-X_L, Bax, or Bak. Whole cell lysates (lanes 1 and 2) are from cells transfected with Bcl-2 (top), Bcl-X_L (second), Bax (third), or no plasmid (bottom) together with either BI-1-Flag plasmid (lane 1) or vector control (lane 2), representing 1/40 of the input for immunoprecipitation.

Bax has been reported to associate primarily with mitochondria in yeast and mammalian cells (Zha et al., 1996). Thus, to the extent that these two proteins reside in different organellar compartments, these observations support the notion of Bcl-2 operating downstream of Bax. However, others have shown that when Bcl-2 is targeted exclusively to the ER through substitution of the C terminus of cytochrome *b5*, it retains the ability to block apoptosis induced by some stimuli (Zhu et al., 1996). Moreover, the adenovirus Bcl-2 homolog E1B 19K can antagonize Bax-induced apoptosis but apparently is not associated with mitochondria (White and Cipriani, 1989; Han et al., 1996). This implies that Bcl-2 and E1B 19K need not necessarily be associated with mitochondria where Bax mostly resides, raising the possibility that Bcl-2 and Bax control parallel pathways that independently provide signals for cell survival and death, respectively. If true, then an alternative interpretation is

that BI-1 is a downstream effector of Bcl-2, which prevents Bax-induced death in yeast by substituting for Bcl-2.

Though mitochondria have received much attention recently for their role in apoptosis, the ER has also been implicated in cell death regulation. The ER performs several essential functions, including protein processing and translocation, vesicle transport, and maintaining calcium homeostasis (reviewed by Teasdale and Jackson, 1996). It has been shown that Bcl-2 can alter regulation of Ca^{2+} in the ER, preventing loss of Ca^{2+} from this organelle following growth factor deprivation and decreasing the basal efflux of Ca^{2+} under normal circumstances (Baffy et al., 1993; Lam et al., 1994). Depletion of intra-ER Ca^{2+} stores has been reported to induce apoptosis (Baffy et al., 1993). Moreover, release of Ca^{2+} into the cytosol can induce mitochondrial permeability transition (PT) pore opening (Bernardi et al., 1994; Kroemer et al., 1996), thus creating a functional connection between the ER and mitochondria. Interestingly, antisense-mediated ablation of one of the ER Ca^{2+} channels, namely the inositol triphosphate-gated receptor-1, has revealed a requirement for this Ca^{2+} channel in T-cell apoptosis induced by diverse stimuli, including anti-Fas antibodies, glucocorticoids, and ionizing radiation (Jayaraman and Marks, 1997). Thus, regulation of Ca^{2+} trafficking through effects on Ca^{2+} channels in the ER represents one of several tenable hypotheses for explaining how a multiple-membrane-spanning protein such as BI-1 might directly or indirectly modulate cell death pathways.

What might BI-1 be doing in intracellular membranes? Based on the predicted multiple transmembrane segments, BI-1 could possibly function as a receptor or an ion-channel protein. Similar to ion-channels, some of the putative transmembrane segments of BI-1 when examined on α -helical wheel plots predict the presence of hydrophilic residues on one face of the α helices rather than uniformly hydrophobic residues like those found in the transmembrane domains of most cell surface growth factor receptors. If several amphipathic helices assembled in the membrane, creating an aqueous lumen ringed by the hydrophilic surfaces of these putative α helices, then BI-1 could potentially form an ion channel in membranes. Alternatively, the apparent physical association of BI-1 and Bcl-2 raises the intriguing possibility that BI-1 and Bcl-2 could create heteromeric channels, with Bcl-2 presumably contributing its 5th and 6th amphipathic α -helical domains, which have been shown to be required for *in vitro* pore formation (Schenkel et al., 1997), and BI-1 providing some of its amphipathic transmembrane α -helical segments. Precedent for this idea exists among some types of K^+ channels in which the functional channel is comprised of a heterotetramer, with each subunit contributing transmembrane α helices that surround a central ion pore (Catterall, 1995). Alternatively, the interaction of Bcl-2 and BI-1 could provide a mechanism by which BI-1 regulates the previously described Bcl-2 channel, or conversely, by which Bcl-2 regulates a hypothetical BI-1 channel, analogous to some voltage-gated Na^+ and Ca^{2+} ion channels in which a single protein forms the actual channel but this channel is highly regulated by associated integral

membrane proteins (Catterall, 1995). Also, by functioning as an ion channel, conceivably BI-1 might alter ion gradients, pH, or voltage (DV) across the membranes where Bcl-2 family proteins reside, thus either inhibiting or enhancing their ability to integrate into membranes and form ion channels (review by Reed, 1997a). It should be noted however that Bcl-2 has been reported to bind multiple proteins (Reed, 1997a) and thus may be promiscuous in its interactions with other proteins, including BI-1. Thus, while the significance of the interaction of BI-1 with Bcl-2 remains to be determined, the data presented here nevertheless establish that BI-1 is a novel suppressor of cell death.

The yeast-based functional cloning strategy employed here provides an alternative approach for identifying proteins that regulate mammalian cell death. Though BI-1 can interact with Bcl-2 (or with a complex of proteins that includes Bcl-2), it is unlikely that interaction cloning methods based on either yeast two-hybrid or λ -phage expression library screening using ligand-blotting would have detected BI-1, because of its hydrophobic characteristics. Protein purification attempts based on ability to interact with Bcl-2 also would have been unlikely to succeed for the same reason. Thus, functional screening for Bax suppressors in yeast provides a powerful approach that complements these other methods and that seems likely to yield new insights into the biochemistry and genetics of mammalian cell death regulation. Future studies, including targeted gene knockout experiments in mice, will better define the overall role played by BI-1 in the Bcl-2/Bax pathway for cell death regulation.

Experimental Procedures

Yeast Methods

Yeast strains used included: BF264-15Dau (*MATa ade1 his2 leu2-3, 112 trp1-1a ura3*) (Lew et al., 1991) and EGY48 (*MAT α trp1 ura3 his3 leu2::plexAop₆-LEU2*). Strain QX95001 is BF264-15Dau containing the *LEU2*-marked mBax-encoding plasmid YEp51-Bax. Strains BF264-15Dau and EGY48 were maintained in the rich YPD medium, and strain QX95001 was maintained in SD-Leu (synthetic dropout medium lacking leucine). Transformations, plasmid extractions, and protein extracts were prepared as described (Sato et al., 1994; Zha et al., 1996).

For cDNA library screening, QX95001 cells were grown to mid-log phase in the SD-Leu liquid medium and transformed with 100 μ g of HepG2 cDNA library DNA by a LiOAc method. Bax-resistant transformants were directly selected on galactose-containing synthetic dropout medium lacking leucine and uracil (SD-leu+ura). An aliquot of transformation mixture was also spread on glucose-containing medium to determine transformation efficiency. Bax-resistant colonies were patched onto galactose-containing SD-leu+ura plates. Con-loss assays were performed as described (Ausubel et al., 1991).

Plasmid Constructions

pcDNA3-hBax and pRc/CMV-Bcl-2 contain human Bax and human Bcl-2 cDNAs in the expression plasmids pcDNA3 and pRc/CMV, respectively. The C-terminal HA-tagged BI-1 plasmid was constructed in two steps. The C terminus of BI-1 (from the internal BamHI site) was first PCR-amplified to add a XhoI site to the very C terminus of the BI-1 ORF (just before the stop codon). PCR primers used were 5'-GGGGATCCATTGGCCTTCCAG and 5'-GGCTCGA GTTTCTCTCTTCTTCTTATCC. The resulting PCR product was digested with BamHI and XhoI and subcloned into pcDNA3 containing an oligonucleotide encoding three in-frame copies of the HA

tag downstream of the XhoI site, producing plasmid pQX9645. Next, the N-terminal portion of BI-1 was re-ligated into pQX9645, giving rise to pcDNA3-BI-1-HA. An EcoRI-XhoI fragment containing BI-1 cDNA from pcDNA3-BI-1-HA was subcloned into pcDNA3 containing a single copy of the FLAG epitope downstream of the XhoI site and into the GFP vector pEGFP-N2 (Clontech Inc.) between the EcoRI and Sall sites. The 2.6 kbp BI-1 cDNA was subcloned in reverse (antisense) orientation between the XhoI and EcoRI sites of pcl-Neo (Invitrogen, Inc.), producing pcl-Neo-BI-1-AS.

Cell Culture and Transfections

293, 293T, GM701, and Cos7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin sulfate and transfected as described (Zha et al., 1996). For cell death assays, 293 cells were seeded at 8×10^5 cells per 60 mm dish and 1 day later transfected with various combinations of plasmids (9 μ g total DNA). Precipitates were removed after 8 hr and replaced with fresh medium. Both floating and adherent cells (after trypsinization) were collected at 24 hr post-transfection and analyzed by trypan blue dye exclusion assay, counting a minimum of 300 cells and performing experiments in triplicate (mean value $\pm$ SD). The transfection efficiency was estimated to be $\geq 70\%$ based on cotransfections with a β -gal reporter plasmid. DAPI staining for assessing nuclear morphology was also performed.

For GM701 fibroblasts, cells were seeded into 6-well tissue culture dishes at 6×10^4 cells/well and cotransfected with 0.5 μ g of β -gal reporter plasmid and 4 μ g of various other expression plasmids. Precipitates were removed 6 hr later, and 18 hr posttransfection cells were washed three times with DMEM and incubated in medium containing 0.1% FBS for 30 hr before fixing and staining with X-gal (Zha et al., 1996).

FL5.12 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 10% WEHI-3B conditioned medium, L-glutamine, and antibiotics. Plasmid DNAs (20 μ g) were introduced into FL5.12 cells by electroporation (GenePulser; Biorad) using 650 V/cm and 1025 μ F, followed by selection in medium containing puromycin (0.5 μ g/ml). Clones expressing BI-1-GFP fusion protein were screened initially by fluorescence microscopy, followed by FACS and immunoblot analysis. IL-3 withdrawal and cell viability determinations were performed as described (Wang et al., 1995). For drug-resistance assays, either etoposide (5 μ g/ml) or staurosporine (0.5 μ M) was added to cells at a density of 5×10^5 cells/ml and incubated for up to 3 days before determining the percentage cell viability based on trypan blue dye exclusion.

Immunoprecipitation and Immunoblot Assays

Cell lysates were prepared using HKME solution (10 mM HEPES [pH 7.2], 142 mM KCl, 5 mM MgCl₂, 1 mM EGTA) containing 1% NP-40 and protease inhibitors (1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 0.7 μ g/ml pepstatin) and cleared by incubation with the rec-protein G-Sepharose 4B (ZYMED). Lysates were then diluted in HKME to a final concentration of 0.6% NP-40 and incubated with anti-Flag M2 antibody (Kodak) at 4°C for 2 hr with constant rotating, followed by rec-protein G beads for 1 hr. Immobilized immunocomplexes were washed four times in HKME containing 0.2% NP-40 before boiling in SDS sample buffer. Cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Biorad). After blocking with 5% non-fat milk, 2% bovine serum albumin (BSA) in TBST (10 mM Tris [pH 7.5], 142 mM NaCl, 0.1% Tween 20) at the room temperature for 2 hr, blots were incubated in the same solution with various primary antibodies, including monoclonal antibodies specific for HA (12 CA5 [0.8 μ g/ml]) or Flag (M2 [3 μ g/ml]) and polyclonal antisera specific for Bcl-2 (0.1% [v/v]), Bcl-X_L (0.1%), Bax (0.1%), CPP32 (0.1%), PARP (0.1%), or F₁- β -ATPase (0.1%), followed by 0.6 μ g/ml horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Biorad) secondary antibodies. Bound antibodies were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham).

In Vivo Cross-Linking

Transfected 293 cells were washed twice with PBS and then incubated for 20 min at room temperature in PBS with 1 mM DTBP

(dimethyl-3,3'-dithiobispropionimidate [2HCl]) (Pierce, Inc.) with gentle shaking. After extensive washing in PBS, cells were lysed in RIPA (10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors, and immunoprecipitations were performed using anti-HA (12CA5) or anti-Flag (M2) monoclonals. Immune complexes were immobilized on protein G beads and washed four times with RIPA, followed by boiling in SDS sample buffer (containing 5% β -mercaptoethanol). Samples were analyzed by SDS-PAGE/immunoblotting using rabbit antisera specific for Bcl-2 or Bax.

Immunofluorescence and Confocal Microscopy

Cells transiently transfected with pcDNA3-BI-1-Flag were trypsinized and seeded into chamber slides. The next day, cells were washed in PBS and fixed in PBS containing 3.7% paraformaldehyde, followed by washing twice in PBS. Cells were then permeabilized in 0.1% Triton X-100/PBS for 20 min and preblocked in PBS containing 3% BSA, 2% FBS, 0.1% goat serum. Anti-Flag antibody M2 (3 μ g/ml) was added to cells in the same solution and incubated for 1 hr, followed by washing three times in PBS/0.1% Triton X-100 and incubation with 2 μ g/ml FITC-conjugated secondary anti-mouse antibody (Dako) for 1 hr. After washing three times in PBS, slides were covered in Vectashield mounting medium (Vector Laboratories, Inc.) and sealed with nail polish. For two-color analysis, GFP-BI-1-transfected cells were incubated with 20 nM Mitotracker (Molecular Probes, Inc.) in normal growth medium for 20 min at 37°C. Confocal fluorescence microscopy was performed using an Axiophot photomicroscope (Zeiss, Inc., Oberkochen, Germany).

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The Mitochondrial F_0F_1 -ATPase Proton Pump Is Required for Function of the Proapoptotic Protein Bax in Yeast and Mammalian Cells

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Summary

The proapoptotic mammalian protein Bax associates with mitochondrial membranes and confers a lethal phenotype when expressed in yeast. By generating Bax-resistant mutant yeast and using classical complementation cloning methods, subunits of the mitochondrial F_0F_1 -ATPase proton pump were determined to be critical for Bax-mediated killing in *S. cerevisiae*. A pharmacological inhibitor of the proton pump, oligomycin, also partially abrogated the cytotoxic actions of Bax in yeast. In mammalian cells, oligomycin also inhibited Bax-induced apoptosis and activation of cell death proteases. The findings imply that an intact F_0F_1 -ATPase in the inner membrane of mitochondria is necessary for optimal function of Bax in both yeast and mammalian cells.

Introduction

Altered function of mitochondria has been recognized for many years as an important contributor to ischemic and necrotic cell death (Bernardi et al., 1994). Recently, however, evidence has accumulated suggesting a critical role for these organelles in apoptosis and programmed cell death (Petit et al., 1996; Hirsch et al., 1997).

Proteins of the Bcl-2 family are important regulators of mammalian cell life and death, with some functioning to prevent and others to promote apoptosis (Reed, 1994; Yang and Korsmeyer, 1996). These proteins can also modulate cell death processes that result in necrosis rather than apoptotic cell death, under some circumstances (Kane et al., 1995; Shimizu et al., 1996). Most Bcl-2 family proteins are integral membrane proteins that reside in the outer mitochondrial membrane, as well as some other intracellular membranes (Krajewski et al., 1993; González-García et al., 1994).

At present, the biochemical mechanism by which Bcl-2 and its homologs regulate cell death remains controversial (Reed, 1997). The three-dimensional structure of one of the Bcl-2 family proteins suggests similarity to the pore-forming domains of certain bacterial toxins, such as diphtheria toxin and the colicins (Muchmore et al., 1996). Moreover, the antiapoptotic proteins Bcl-2

and Bcl-X_L as well as the proapoptotic protein Bax can form ion channels in synthetic membranes in vitro in a pH-dependent manner (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). However, Bcl-2 and Bcl-X_L also clearly have apoptosis-regulatory functions apart from their ability to form channels, in that they bind to several other proteins that can modulate responses to apoptotic stimuli (Reed, 1997). Bcl-2 has been shown to protect mitochondria from loss of membrane potential and release of caspase-activating proteins such as cytochrome c and apoptosis-inducing factor (AIF) (Susin et al., 1996; Zamzami et al., 1996; Kluck et al., 1997; Yang et al., 1997), whereas Bax can induce loss of mitochondrial membrane potential and activation of caspases (Xiang et al., 1996; Jürgensmeier et al., 1997). It remains unclear whether these effects of Bcl-2 and Bax on mitochondrial physiology are a direct result of their intrinsic activities as channel proteins, which conceivably may transport either ions or proteins, as opposed to an indirect consequence of their effects on other channel proteins associated with mitochondrial membranes.

When expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe*, the proapoptotic mammalian protein Bax confers a lethal phenotype (Sato et al., 1994; Ink et al., 1997; Jürgensmeier et al., 1997). Yeast cells undergoing Bax-induced death exhibit ultrastructural changes that include massive cytosolic vacuolization and apparent disruption of mitochondria (Ink et al., 1997; Jürgensmeier et al., 1997), similar to mammalian cells that express Bax in the presence of caspase inhibitors (Xiang et al., 1996). In yeast, the Bax protein is associated primarily with mitochondria, and the targeting of Bax to these organelles appears to be important for its lethal phenotype in yeast (Zha et al., 1996). Similar to mammalian cell apoptosis, expression of Bax in yeast has been reported to induce release of cytochrome c from mitochondria into the cytosol (Manon et al., 1997). Yeast cell death induced by Bax or the closely related Bak protein can be specifically suppressed by antiapoptotic Bcl-2 family proteins (Sato et al., 1994; Bodrug et al., 1995; Hanada et al., 1995; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). Further evidence of similarities in at least some of the mechanisms by which Bax functions in animal cells and yeast comes from the observation that cell death induced by Bax can be suppressed in both mammalian cells and *S. cerevisiae* by BI-1, a human protein that contains multiple membrane-spanning domains (Xu and Reed, 1998 [this issue of *Molecular Cell*]).

The functions of Bcl-2 family proteins are often conserved across evolution, with the human Bcl-2 protein, for example, exhibiting potent antiapoptotic activity even in nematodes and insect cells (Alnemri et al., 1992; Vaux et al., 1992; Hengartner and Horvitz, 1994). Moreover, the human Bcl-2 protein has been reported to protect superoxide dismutase (sod)-deficient strains of budding yeast from cell death induced by oxidative stress (Kane et al., 1993), implying an evolutionarily

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conserved function perhaps even in some unicellular organisms. Though programmed cell death (PCD) is conventionally thought to operate only in multicellular organisms, recent studies have revealed apoptosis-like cell death in several unicellular eukaryotes, including *Dictyostelium discoideum* (Cornillon et al., 1994), *Trypanosoma brucei rhodesiense* (Welburn et al., 1996), *Trypanosoma cruzi* (Ameisen et al., 1996), *Leishmania amazonensis* (Moreira et al., 1996), and *Tetrahymena thermophila* (Christensen et al., 1995). It has also been suggested that certain forms of PCD may even exist in prokaryotes (Ameisen, 1996), where cell suicide mechanisms could potentially limit spread of viruses, reduce competition for nutrients during times of starvation, or ensure that cells with damaged DNA do not pass their defective genomes on to future generations. Reasoning that at least some of the functions of Bcl-2 family proteins appear to be conserved in yeast, we undertook a classical genetics approach designed to identify yeast genes that are required for Bax-mediated lethality in *S. cerevisiae*.

Results

Creation of a Mutant Yeast Strain that Displays Resistance to Bax-Induced Cell Death

Yeast strain EGY48 was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) and then transformed with YEp51-Bax, a *LEU2*-marked, high-copy episomal plasmid that produces the mouse Bax protein under the control of the galactose-inducible *GAL10* promoter (Zha et al., 1996). Transformants were plated on leucine-deficient semisolid medium containing galactose, resulting in ~50 viable colonies. To exclude clones that might have survived because of defects in transactivation of the *GAL10* promoter in YEp51-Bax, these transformants were cured of the YEp51-Bax plasmid and then retransformed with the plasmid pEG202-Bax in which Bax is produced from a strong constitutive *ADH1* promoter. The resulting 24 Bax-resistant clones were mated with wild-type Myy290 strain cells, yielding 9 diploids in which sensitivity to Bax-mediated killing was restored, thus suggesting a recessive mutation. Tetrad analysis was then performed for these recessive mutants, with only one (hereafter designated as Bax-resistant mutant-1 [BRM1]) exhibiting 2:2 Mendelian segregation of the Bax-resistance phenotype in a manner consistent with a single gene defect (Figure 1A).

The genomic mutation in BRM1 cells did not interfere with Bax protein production, as determined by immunoblotting (Figure 1B). For these experiments, the wild-type and BRM1 cells were transformed with pGilda-Bax, which produces Bax as a fusion protein with a portion of LexA (used analogous to an epitope tag here), or the pGilda plasmid, which produces only the LexA fragment. The addition of the LexA tag to Bax does not interfere with its intracellular targeting or cell death-inducing function in yeast (Zha et al., 1996).

Complementation Cloning of Yeast Genes that Restore Sensitivity to Bax

The BRM1 cells containing pGilda-Bax were transformed with a centromere-based yeast genomic library.

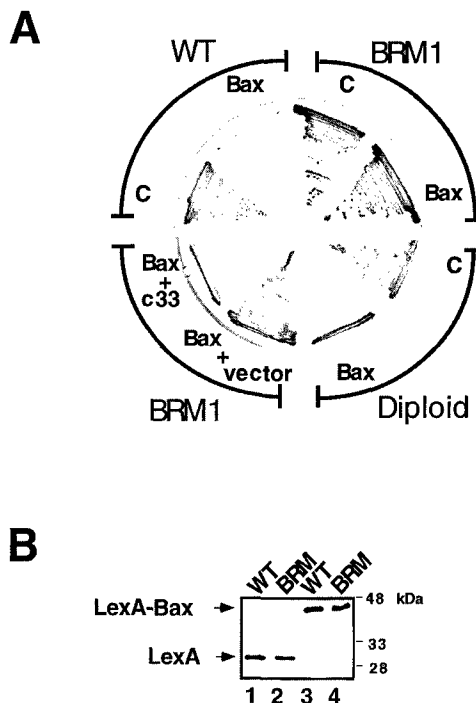


Figure 1. Generation of a Bax-Resistant Yeast Mutant and Identification of Genomic Clones that Restore Bax Sensitivity

(A) Wild-type EGY48 yeast (WT), Bax-resistant-mutant (BRM1), and diploid cells derived from mating Myy290 (wild-type) $\times$ BRM1 were transformed with control plasmid pGilda (C) or galactose-inducible pGilda-Bax (Bax). BRM1 was also cotransformed with pGilda-Bax and clone 33 from yeast genomic library or control vector YCp50. Each transformant was first grown on glucose-based plate and then a colony was restreaked on galactose-containing plates and incubated at 30°C for 4 days.

(B) Immunoblot analysis is shown for lysates (10 μ g) derived from WT yeast (lanes 1 and 3) and BRM1 (lanes 2 and 4) cells transformed with pGilda (produces LexA protein DNA-binding domain without a nuclear localization sequence) (lanes 1 and 2) or pGilda-Bax (produces LexA-Bax fusion protein) (lanes 3 and 4). Antigens were detected using anti-LexA rabbit antiserum.

Eight transformants were identified by replica plating that appeared to have a restoration of their sensitivity to Bax-mediated cell death. The plasmids recovered from these eight transformants were then retransformed with pGilda-Bax into BRM1 cells, with only one of these clearly restoring sensitivity to Bax-mediated lethality to wild-type levels (Figure 1A). DNA sequence analysis revealed that this clone contained three genes, including *ATP4*, which encodes subunit 4 of the yeast F_0F_1 -ATPase, a proton pump located in the inner membrane of mitochondria (Weber and Senior, 1997).

ATP4 Is Required for Bax-Induced Lethality in Yeast

Since it has been previously suspected that Bax promotes cell death at least in part through effects on mitochondria (Xiang et al., 1996; Zha et al., 1996), we focused on *ATP4* as a likely candidate gene that is required for Bax-induced killing of yeast. The *ATP4* gene has been previously disrupted in *S. cerevisiae* by *URA3* insertional mutagenesis (Velours et al., 1989; Paul et al., 1992), thus

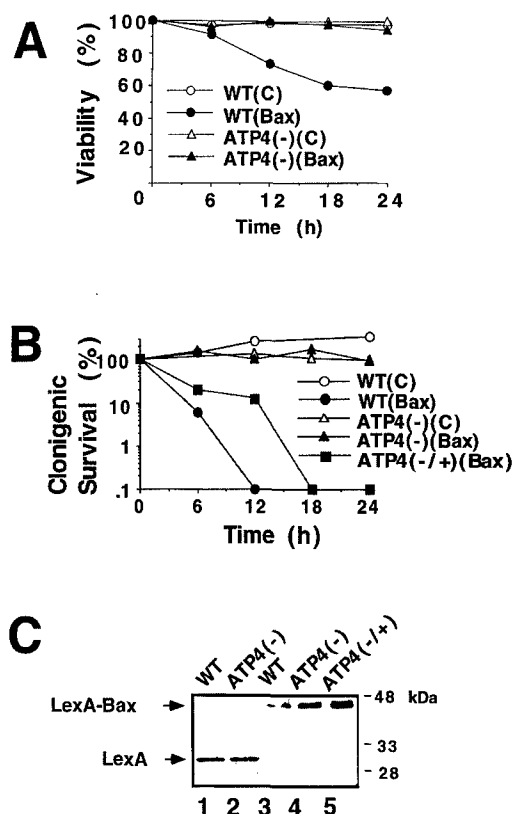


Figure 2. Yeast with Disrupted *ATP4* Gene Are Resistant to Bax-Induced Cell Death

Wild-type D273-10B/A yeast, *ATP4* knockout yeast strain PVY10 (*ATP4*⁻), and heterozygous diploids derived from mating of PVY10 with Myy290 (*ATP4*^{-/+}) were transformed with pGilda (C) or pGilda-Bax. The cells were grown in glucose-based medium and then recultured in galactose-based medium to induce protein expression from the *GAL1* promoter in pGilda plasmids.

(A) The percentage of trypan blue dye excluding cells was determined at various times after switching to galactose-based media (mean $\pm$ SE; $n = 3$; SE bars are obscured by symbols).

(B) Clonogenic survival was determined by recovering cells at various times from galactose-containing medium and plating 1000 cells on glucose-based semisolid medium. Data are representative of at least three experiments.

(C) Immunoblot analysis was performed to assess LexA and LexA-Bax protein levels in cells after 12 hr of culture in galactose-based media, as described for Figure 1.

creating the *ATP4*-deficient strain PVY10. We therefore tested PVY10 cells for resistance to Bax. For these experiments, PVY10 cells were transformed with pGilda-Bax or pGilda control plasmid and grown for various times in galactose-containing medium to induce expression of Bax, and cell viability was monitored by trypan blue dye exclusion. *ATP4*-deficient PVY10 cells were not killed after switching from glucose- to galactose-containing medium (Figure 2A). In contrast, cells of the isogenic wild-type strain (D273-10B/A) that had been transformed with pGilda-Bax began to die within 12 hr after switching to galactose.

To further verify that *ATP4*-deficient PVY10 cells are resistant to Bax-mediated lethality, a clonogenic survival assay was performed in which cells were switched from glucose- to galactose-containing liquid medium for 1

day to induce expression of Bax, and then plated on glucose-based semisolid medium, which suppresses the *GAL1* promoter in pGilda. The plating efficiency of *ATP4*-deficient PVY10 cells was essentially the same, regardless of whether they contained the pGilda-Bax or pGilda plasmids (Figure 2B). In contrast, colony formation by the isogenic wild-type strain was markedly reduced in cells harboring the pGilda-Bax plasmid compared to the control pGilda vector. Clonogenic survival of the wild-type cells began to decline after as little as 6 hr of exposure to galactose, with essentially all cells failing to form viable colonies after a 12 hr exposure. The differences in the kinetics of loss of clonogenic survival and development of trypan blue positivity (compare Figures 2A and 2B) have been observed previously in Bax-expressing yeast (Zha et al., 1996; Jürgensmeier et al., 1997), and likely reflect a commitment to cell death even before loss of plasma membrane integrity. Mating the PVY10 cells with wild-type haploids to create *ATP4*^{+/+} heterozygous diploids restored sensitivity to Bax. Immunoblot analysis demonstrated that the *ATP4* mutation did not prevent production of Bax protein (Figure 2B). We conclude therefore that *ATP4*, which is a nuclear gene that encodes subunit 4 of the yeast mitochondrial *F*₀*F*₁-ATPase proton pump, is required for Bax-mediated killing of yeast.

The Proton-Pump Inhibitor Oligomycin Inhibits Bax-Induced Killing of Yeast

Oligomycin binds to the *F*₀ portion of the yeast and mammalian *F*₀*F*₁-ATPases and prevents the proton pump from transporting H⁺ ions, thus effectively shutting it off (Tzagoloff, 1970). We reasoned that if the proton pump is required for Bax-mediated lethality in yeast, oligomycin should render wild-type yeast resistant to Bax. To test this hypothesis, D273-10B/A strain yeast that had been transformed with either pGilda-Bax or pGilda control plasmids were cultured for various times in galactose-containing medium with or without oligomycin. Oligomycin did not inhibit the growth of yeast under these conditions due to their ability to produce sufficient ATP from anaerobic fermentation (not shown). Oligomycin is non-toxic in yeast and it fails to induce permeability transition pore (PTP) opening in yeast mitochondria (Jung et al., 1997). As shown in Figure 3, oligomycin partially inhibited the Bax-induced killing of yeast, without interfering with production of the LexA-Bax protein. Thus, similar to disruption of the *ATP4* gene, a pharmacological inhibitor of the *F*₀*F*₁-ATPase proton pump suppresses Bax-mediated lethality in yeast.

Respiration Is Not Required for Bax-Induced Killing of Yeast

It was possible that the genetic disruption or pharmacological inhibition of the *F*₀*F*₁-ATPase proton pump indirectly suppressed Bax-mediated lethality in yeast by blocking respiration (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994). To address this question and to further explore the importance of the *F*₀*F*₁-ATPase, we compared the effects of Bax expression on the viability and clonogenic survival of *rho*⁻ yeast as well as on an additional mutant strain of yeast in which the

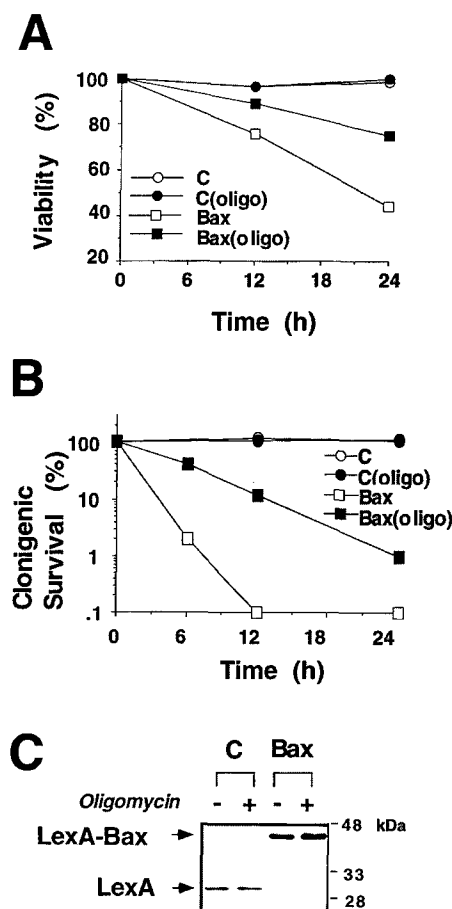


Figure 3. F_0F_1 -ATPase Proton-Pump Inhibitor, Oligomycin, Attenuates Bax-Induced Cell Death in Yeast

Yeast strain D273-10B/A cells transformed with pGilda (C) or pGilda-Bax were initially cultured in glucose-based media, then switched to galactose-containing medium with (closed symbols) or without (open symbols) 10 μ M oligomycin, and the percentage of trypan blue dye excluding cells was determined at various times thereafter (A) or cells were recovered and either 1000 or 3000 cells were plated on glucose-containing semisolid medium (B) (mean $\pm$ SE; $n = 3$; SE symbols are obscured by symbols). In (C), protein lysates (10 μ g) were generated from the same cells after 12 hr of culture in galactose-based medium and analyzed by immunoblotting using anti-LexA antiserum. Lanes 1/2 and lanes 3/4 represent cells containing pGilda or pGilda-Bax, respectively, grown with or without oligomycin as indicated.

δ subunit of F_0F_1 -ATPase had been inactivated by *URA3* insertional mutagenesis (Giraud and Velours, 1994). Unlike the *ATP4* mutant, both *rho*⁻ and *ATP* δ -deficient yeast are *petites*. *Rho*⁻ yeast fail to express all proteins encoded in the mitochondrial genome, and thus lack certain proteins that are critical for respiration. Though certain subchains of the F_0F_1 -ATPase proton pump are encoded in the mitochondrial genome, its activity remains partially functional in *rho*⁻ yeast (Schatz, 1968; Giraud and Velours, 1994). In contrast, loss of the nuclear-encoded *ATP* δ protein results in deficient function of both the F_0 and F_1 components of the proton pump (Giraud and Velours, 1997). These two *petite* strains grew at comparable rates in the absence of Bax (not shown).

The *rho*⁻ yeast transformed with pGilda-Bax began

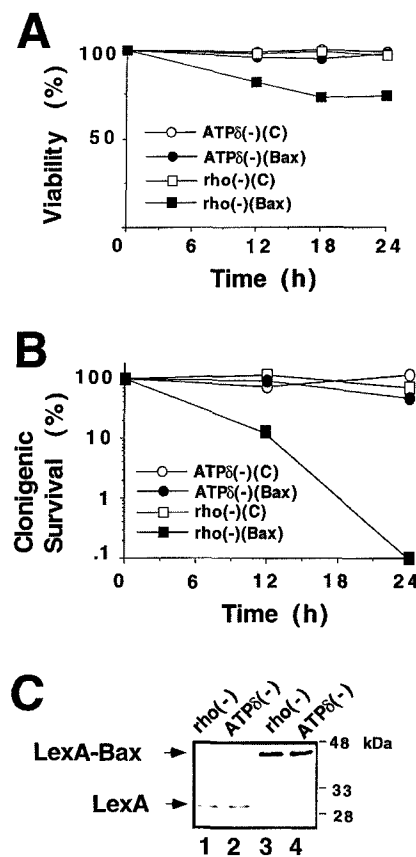


Figure 4. *ATP* δ -Deficient but Not *rho*⁻ *petite* Yeast Are Bax-Resistant

Strain CGY1 yeast that harbor a *URA3*-disrupted *ATP* δ gene and *rho*⁻ yeast (both *petite*) were transformed with pGilda (C; open symbols) or pGilda-Bax (closed symbols). Cell viability (A), clonogenic survival (B), and Bax protein levels (C) were measured as described for Figure 2 at various times after switching cells to galactose-containing medium (mean $\pm$ SE; $n = 3$; some SE symbols are obscured by symbols). In (C), lanes 1/2 and lanes 3/4 represent *rho*⁻ and *ATP* δ -deficient yeast transformed with pGilda and pGilda-Bax, respectively.

to die when switched to galactose-containing medium, whereas *rho*⁻ cells containing the control pGilda vector did not (Figure 4A). Note however that the Bax-induced cell death and loss of clonogenic survival occurred with delayed kinetics relative to wild-type yeast (compare with Figure 2). Thus, the absence of respiration in *rho*⁻ cells may reduce but does not abrogate Bax-mediated lethality in yeast. In contrast, yeast lacking the δ subunit of the F_0F_1 -ATPase were completely resistant to Bax, despite expressing LexA-Bax protein at levels equivalent to those of the *rho*⁻ cells (Figure 4). We conclude therefore that respiration is not required for Bax-induced killing of yeast, but the F_0F_1 -ATPase proton pump is.

Oligomycin Also Inhibits Bax-Induced Apoptosis and Activation of Cell Death Proteases in Mammalian Cells

No mammalian cells exist that harbor mutations within subunits of the mitochondrial F_0F_1 -ATPase. Thus, to explore whether the proton pump is also required for optimal function of Bax in mammalian cells, one is limited

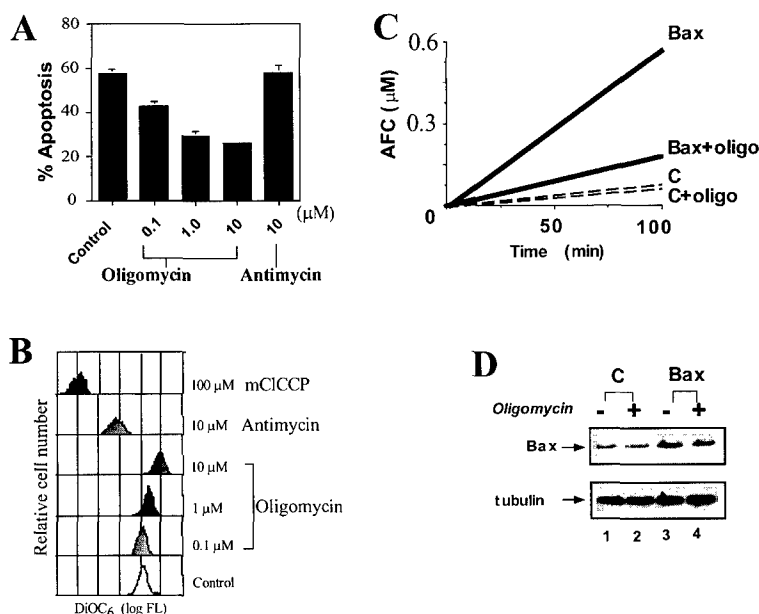


Figure 5. The F_1F_0 -ATPase Inhibitor Oligomycin Suppresses Bax-Induced Apoptosis and Caspase Activation in 293T Cells

(A) 293T cells were cultured in DMEM-high glucose medium to maintain ATP supplies by glycolysis. Four hours after transfection with 9 μ g of pcDNA-Bax (Bax) or control pcDNA (C) plasmids with 1 μ g of pEGFP, the culture medium was exchanged with fresh medium containing or lacking 0, 0.1, 1, or 10 μ M oligomycin or 10 μ M antimycin A. After an additional 8 hr of culture, the cells were collected. In (A), the percentage of GFP⁺ cells with apoptotic morphology was determined by DAPI-staining (mean $\pm$ SD; $n = 3$).

(B) 293T cells were recovered from cultures of untransfected cells and incubated with DiOC₆, followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC₆ labeling, an aliquot of the control untreated cells was exposed to the protonophore mCICCP for 15 min prior to incubation with DiOC₆.

(C) Lysates derived from cells that had been cultured with or without 10 μ M of oligomycin

were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1997). Typical substrate hydrolysis progress curves are shown (representative of three experiments).

(D) Aliquots of the same lysates employed for caspase assays were subjected to immunoblot analysis, employing anti-hu Bax antiserum with ECL-based detection. The blot was subsequently reprobed with anti-tubulin antibody to verify loading of equivalent amounts of total protein.

to pharmacological studies employing oligomycin. In mammalian cells, unlike in yeast, oligomycin is toxic and leads secondarily to mitochondrial PTP opening and either apoptosis or necrosis, depending on the particular cells and circumstances evaluated (Castedo et al., 1996). Cell death caused by oligomycin, however, can be delayed by culturing in high glucose-containing medium, which helps to maintain ATP levels via glycolysis (Eguchi et al., 1997; Leist et al., 1997). We therefore explored the effects of oligomycin in human 293T kidney epithelial cells grown in high glucose medium, using a transient Bax transfection assay to induce apoptosis and activation of cell death proteases (caspases) (Zha et al., 1996; Deveraux et al., 1997; Jürgensmeier et al., 1997). For all experiments, oligomycin was added 4 hr after transfections; caspase activity and apoptosis were then measured after an additional 8 hr of culture. Thus, the experiments were performed within the first 12 hr after Bax transfections, before oligomycin caused cell death (>90% trypan blue dye exclusion in control cultures).

As shown in Figure 5A, oligomycin reduced the percentage of apoptotic cells in cultures of Bax-transfected 293T cells in a dose-dependent manner, with concentrations of 1–10 μ M oligomycin preventing approximately half of the Bax-induced cell death. DiOC₆-based measurement of mitochondrial membrane potential demonstrated that these concentrations of oligomycin resulted in hyperpolarization of mitochondria in 293T cells, consistent with a block of the proton pump causing accumulation of H⁺ ions in the intermembrane space of these organelles (Figure 5B). Acute exposure of the cells to the protonophore mCICCP confirmed that the DiOC₆ staining was dependent on the mitochondrial pH gradient (Figure 5B), thus verifying the specificity of this assay.

In contrast to oligomycin, culturing Bax-transfected

293T cells with the respiratory complex III inhibitor antimycin A did not impair Bax-induced apoptosis under these conditions, but did markedly reduce mitochondrial $\Delta\Psi$ (Figure 5B). These findings in mammalian cells thus support the observations obtained with yeast, demonstrating again that respiration is unnecessary for Bax-mediated cell death.

Since gene transfer-mediated overexpression of Bax has been shown to induce activation of caspases that can cleave the substrate peptide DEVD (Deveraux et al., 1997; Jürgensmeier et al., 1997), we measured the effects of oligomycin treatment on Bax-induced activation of DEVD-cleaving caspases using lysates from the transfected 293T cells. As shown in Figure 5C, 293T cells transfected with pcDNA3-Bax contained markedly elevated levels of caspase activity compared to control transfected cells. Addition of 1–10 μ M oligomycin to the cultures substantially reduced the amount of Bax-induced caspase activity (Figure 5C and data not shown).

Under these same conditions, ATP levels were maintained to within ~95% of control levels for 293T cells treated with 1 μ M oligomycin (32 ± 3 nmol/mg protein versus 34 ± 3 nmol/mg protein) and to within ~75% of control levels for cells treated with 10 μ M oligomycin (25 ± 2 nmol/mg protein). Thus, the oligomycin-mediated protection against Bax-induced apoptosis cannot be ascribed to reduced ATP levels. Oligomycin also did not impair production of the expected 21 kDa Bax protein in 293T cells (Figure 5D).

When used at high concentrations in vitro, oligomycin has been reported to inhibit the plasma membrane Na-K ATPase ($IC_{50} \sim 5$ μ M) (Decottignies et al., 1995). We therefore tested the effect of the Na-K ATPase inhibitor ouabain on Bax-induced apoptosis in 293T cells, but found that even at 100 μ M, ouabain had no influence on Bax function (not shown). Based on the above results,

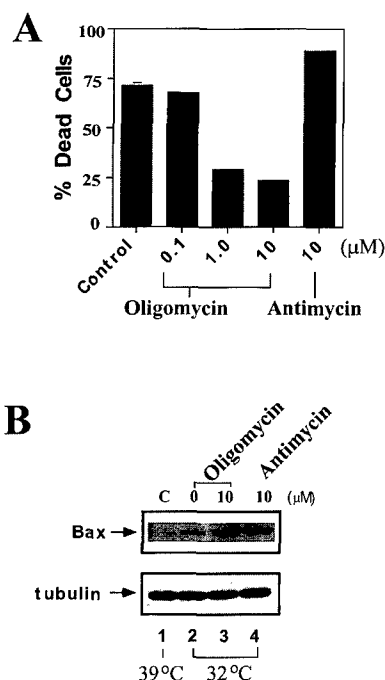


Figure 6. Oligomycin Inhibits p53-Induced Apoptosis

BRK cells that contain ts-p53 (Han et al., 1996) were maintained at a nonpermissive temperature of 39°C, then cultured at 32°C to induce p53 in the presence or absence of 0.1–10 μ M oligomycin or 10 μ M antimycin A. In (A), the percentage of dead cells was determined by DAPI staining 12 hr after shifting to 32°C (mean $\pm$ SD; $n = 3$). In (B), Lysates were prepared from BRK cells that had been cultured at 39°C (lane 1) as a control (C) or at 32°C (lanes 2–4) without or with 10 μ M oligomycin or 10 μ M antimycin for 12 hr, normalized for total protein content (5 μ g/lane), and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobe with anti-tubulin.

therefore, we conclude that the F_0F_1 -ATPase proton pump is either required for optimal function of Bax in 293T cells or enhances Bax's ability to induce apoptosis in these human cells.

Oligomycin Inhibits p53-Induced Apoptosis

The induction of apoptosis in baby rat kidney (BRK) cells by p53 has been shown to be Bax-dependent (Han et al., 1996; Sabbatini et al., 1997). We therefore employed BRK cells that express a temperature-sensitive mutant of p53, and examined the impact of culturing these cells with various concentrations of oligomycin at either the permissive temperature of 32°C where p53 is active and apoptosis ensues or at the nonpermissive temperature of 37–39°C where p53 is inactive. As in the prior experiments, these cells were grown in high glucose medium to maintain ATP levels through glycolysis.

Oligomycin reduced the percentage of apoptotic cells by approximately two-thirds when ts-p53 BRK cells were cultured at 32°C to active p53 (Figure 6A). Under these same conditions, ATP levels were maintained at $\sim$ 100% of control levels in BRK cells treated with 1 μ M oligomycin and to within $\sim$ 70% of control for 10 μ M oligomycin (not shown). In contrast, antimycin A had no apparent effect on p53-induced apoptosis in BRK cells (Figure 6A), yet reduced ATP levels more than oligomycin. Immunoblot analysis of BRK cells demonstrated

that neither oligomycin nor antimycin A (10 μ M) impaired p53-induced expression of Bax when the cells were cultured at the permissive temperature of 32°C (Figure 6B). Taken together, therefore, these data provide further evidence that Bax-dependent apoptosis requires the mitochondrial F_0F_1 -ATPase proton pump.

Discussion

Here we present genetic evidence that the mitochondrial F_0F_1 -ATPase proton pump is required for Bax-induced cell death in yeast. By employing oligomycin, a specific inhibitor of the proton pump, we also found that mitochondrial F_0F_1 -ATPase is apparently required for at least optimal induction of apoptosis and activation of caspases by Bax in mammalian cells. Based on currently available information, Bax and the F_0F_1 -ATPase proton pump are thought to reside in different mitochondrial membranes, with Bax associated with the outer membranes oriented primarily toward the cytosol and the F_0F_1 -ATPase in the inner membrane (Figure 7). Presumably, therefore, these proteins do not physically interact, though the tendency of Bcl-2 family proteins to concentrate at the junctional complexes of mitochondria, where the inner and outer membranes come into contact (Krajewski et al., 1993; de Jong et al., 1994), may create opportunities for a direct interaction. This might be particularly true when Bax is integrated into membranes within its capacity as a channel-forming protein, which is speculated to involve the insertion of the predicted 5th and 6th α helices of Bax through the lipid bilayer (reviewed in Reed, 1997).

Barring a direct physical interaction, how then might the F_0F_1 -ATPase contribute to Bax-induced cell death? At least two potential explanations can be entertained. First, loss of the proton pump might prevent Bax from integrating into the outer membrane to form a channel. In this regard, the channels formed in vitro by Bax, as well as by Bcl-2 and Bcl-X_L, are voltage-dependent and their activities are modulated by pH (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). For example, the conductances of Bax channels in planar bilayers are reportedly higher at neutral than at acidic pH, whereas Bcl-2 channels open more frequently at lower pH. In addition, most Bcl-2 family proteins including Bax contain a glutamic acid residue between the α 5 and α 6 helices. Protonation of this residue at lower pHs therefore could destabilize the membrane-inserted channel-forming conformation, allowing these two helices to more easily slip back out of the planar bilayer. Thus, alterations of the voltage potential across the inner mitochondrial membrane where the F_0F_1 -ATPase resides or changes of pH at the surface of the inner membrane theoretically could interfere with Bax channel activity, assuming Bax can be influenced by the local voltage potential and pH gradient, particularly at the junctional complexes. The insertion of the α 5 and α 6 helices of Bax into membranes could also provide a way for exposing the BH3 domain of Bax so that the hydrophobic face of this α helix is available for dimerization with Bcl-2 family proteins (Figure 7), as revealed by recent structural studies (Sattler et al., 1997). BH3 domain-mediated dimerization could play an important role in

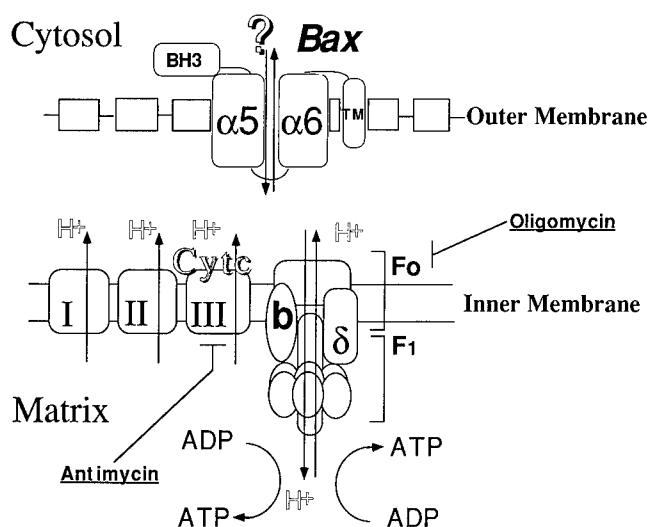


Figure 7. Schematic of Potential Relations between Mitochondrial F_0F_1 -ATPase Proton Pump and Bax

The diagram depicts mitochondria, showing the inner and outer membranes. The outer membrane is thought to be porous because of porin. Bax in the outer membrane is anchored via a C-terminal transmembrane (TM) domain. During channel formation, the predicted 5th and 6th α helices of Bax are speculated to penetrate the lipid bilayer. The BH3 domain ($\alpha 2$ helix) is located on the cytosolic side of the membrane and can mediate dimerization with Bcl-2 and related proteins. The F_0F_1 -ATPase proton pump resides in the inner membrane. The β (equivalent to subunit 4 in yeast) and δ subunits are indicated. The β and δ subunits of F_0 and F_1 , respectively, play important roles in connecting the F_0 proton channel and F_1 ATPase portions together. The transport of H^+ ions by the proton pump is reversible, and can either consume or generate ATP. Oligomycin shuts off the pump, such that protons cannot be transported in either direction. The respiratory chain complexes I, II, and III extrude protons into the intermembrane space. Complex III is inhibited by antimycin A and associated with cytochrome c.

modulating interactions of Bcl-2 and Bcl-X_L with CED-4 or other proteins (Reed, 1997), irrespective of ion-channel formation, at least in mammalian cells.

A second possible explanation for why the F_0F_1 -ATPase is required for Bax-mediated lethality in yeast is that the proton pump may be a downstream effector of Bax. In this regard, the F_0F_1 -ATPase can operate in both forward and reverse directions, either transporting protons into the matrix down their concentration gradient and creating ATP, or pumping protons out of the matrix while consuming ATP (Figure 7). It is conceivable, therefore, that Bax channels render the outer membrane more porous, causing a faster dissipation of the proton gradient than usual through leakage of H^+ ions into the cytosol. As a secondary consequence, the F_0F_1 -ATPase proton pump would be predicted to run in reverse, thus consuming ATP and alkalinizing the matrix by extruding protons. Since alkalization of the matrix has been shown to cause opening of the mitochondria permeability transition (PT) pore in both mammalian and yeast mitochondria (Bernardi et al., 1994; Icha et al., 1997; Jung et al., 1997), the F_0F_1 -ATPase could theoretically facilitate Bax-induced cell death by this mechanism. In this regard, yeast mitochondria have been reported recently to have evidence of a PT pore that can be induced to open by matrix alkalinization (Jung et al., 1997).

In previous studies where oligomycin was used under conditions designed to maintain ATP levels and prevent necrosis, it was observed that cell death induced by etoposide and dexamethasone was inhibited (Eguchi et al., 1997; Leist et al., 1997). In contrast, apoptosis induced by anti-Fas antibody is apparently not dependent on the F_0F_1 -ATPase, since Fas can still induce apoptosis in cells exposed to oligomycin in high-glucose media. Though this observation clearly suggests that the F_0F_1 -ATPase is not absolutely necessary for apoptosis, it

does not discount the possibility that the proton pump contributes to Bax-induced cell killing since Fas-induced apoptosis appears to be relatively Bax-independent (reviewed in Vaux and Strasser, 1996) whereas apoptosis induced by etoposide and dexamethasone can be assisted by Bax (Brady et al., 1996).

In summary, the data reported here demonstrate a role for the mitochondrial F_0F_1 -ATPase in Bax-induced cell death. Though other explanations are possible, we suspect that the functional interaction between Bax and the proton pump is a manifestation of Bax's ability to function as a channel protein in mitochondrial membranes. However, Bax can promote cell death in mammalian cells by at least two mechanisms: (a) by forming channels in membranes; and (b) by dimerizing with anti-apoptotic Bcl-2 family proteins and thereby interfering with their actions independent of channel formation (Reed, 1997). In yeast, we hypothesize that the bioactivity of Bax is entirely dependent on its ability to form channels, since these simple unicellular organisms appear to lack Bcl-2 and CED-4 homologs. In mammalian cells, however, both mechanisms are likely to be operative. It remains to be determined which of these two mechanisms for Bax-mediated cell death (channel formation versus Bcl-2 antagonist) is quantitatively more important in mammalian cells, but cellular context and the specific cell death stimulus involved are likely to be highly important. Regardless, the data presented here suggest that applications of yeast genetics may provide novel insights into the channel-dependent mechanisms of Bax-induced cell death.

Experimental Procedures

Plasmids

YEp51-Bax, pEG202-Bax, and pcDNA-Bax have been described (Zha et al., 1996). The Bax cDNA from pEG202-Bax was subcloned

into the EcoRI and XhoI sites of pGilda (gift of C. Kaiser [MIT]). The YCp50 plasmid (ATCC37419) and yeast genomic library (ATCC37415) were obtained from American Type Culture Collection (Rockville, Maryland).

Yeast Methods

Yeast strains and plasmids used for these studies have been described previously (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994; Zha et al., 1996). For generation of Bax-resistant mutant yeast, EGY48 strain was mutagenized with MMNG (Sigma, Inc.) using routine methods (Guthrie and Fink, 1991). After treatment with MMNG for 10 min, yeast cells were grown for 4 hr in YPD media and then transformed by a LiOAc method with the GAL10 promoter-containing plasmid YEp51-Bax and plated on minimal medium supplemented with required amino acid (MM-A) containing 1% raffinose and 2% galactose. Surviving clones were picked from plates and grown in MM-A with glucose prior to transformation with the ADH1 promoter-containing plasmid pEG202-Bax (Sato et al., 1994) and selection on MM-A/2% glucose plates. Resistant mutants were mated with Myy290 strain (*Mata*, *his3*, *ura3*, *leu2*), and the resulting diploid cells were subjected to tetrad analysis (Guthrie and Fink, 1991). BRM1 cells displayed precisely 50% inheritance of the Bax-resistant phenotype.

Complementation Cloning

BRM1 cells were transformed with the GAL1 promoter-containing plasmid pGilda-Bax and grown in MM-A with glucose to a density of $2-4 \times 10^7$ cells/ml. These cells were then transformed using a LiOAc method with 2 μ g of a yeast genomic library in YCp50 (ATCC) and 20 μ g of salmon sperm DNA (transformation efficiency 1×10^4 to 2×10^4 per μ g DNA). Transformed cells were first plated on MM-A with glucose and then replica-plated to MM-A with galactose. From $\sim 2 \times 10^4$ independent colonies tested, 8 clones were identified that did not grow on MM-A/galactose plates. Plasmids were recovered from these 8 candidates, and BRM1 cells were retransformed with these library plasmid together with pGilda-Bax, thus confirming restoration of sensitivity to Bax to approximately the same level as wild-type yeast for 1 of them. The ends of this plasmid were sequenced by primers flanking the cloning site in YCp50 (5'-CGATCATGCGACCA CACCCGTCCT-3' and 5'-GGTGATGCCGCCACGATGCGTCCG-3'). The DNA sequence results were compared with the Yeast Genomic Data Base using dbFAST (Stanford University).

Yeast Cell Viability Assays

Single colonies of yeast cells transformed with pGilda or pGilda-Bax were grown in 10–20 ml of MM-A/glucose with vigorous aeration at 30°C to an optical density of 0.4–0.5 OD₆₀₀ nm. Cells were pelleted by centrifugation (1000 $\times$ g) for 10 min and washed three times in MM-A/galactose before resuspending in 20 ml of MM-A/galactose and culturing half with 10 μ M oligomycin and half with ethanol solvent control (final 0.1 %). Oligomycin or ethanol was added to MM-A/galactose medium every 12 hr to ensure maintenance of adequate levels of drug. After culturing for various times at 30°C, a 0.5 ml aliquot of cells was removed for trypan blue dye exclusion assay, counting at least 300 total (live and dead) cells. Alternatively, the total cell density of cultures was determined, and either 1000 or 3000 cells were spread on MM-A/glucose plates, followed by culturing at 30°C for 4 days. The number of colonies on plates from the 0 hr cultures was designated as 100%.

Mammalian Cell Transfections and Apoptosis Assays

293T cells were cultured for 12 hr in DMEM-high glucose (4500 mg glucose/l) medium supplemented with 10% fetal bovine serum (FBS) at a density of 10^6 cells in 3.6 ml of medium per 6 cm diameter dish or 3×10^6 cells in 10 ml per 10 cm dish. Fresh medium was exchanged and 4 hr later the cells were transfected with 10 μ g of pcDNA3-Bax versus parental pcDNA3 plasmid (10 cm dishes) or were cotransfected with 1 μ g of pEGFP (Clontech Laboratories, Inc.) and either 9 μ g of pcDNA3-Bax or pcDNA3 control plasmid (6 cm dishes). Four hours after the transfection, the medium was changed with fresh media containing 10 μ M oligomycin, 10 μ M antimycin A, or 0.1% ethanol (solvent). After culturing for an additional 8 hr, both the floating and attached cells were harvested. Half

of the recovered cells were used for immunoblot assays and the remainder were used for either caspase activity assays (Deveraux et al., 1997) or for DAPI staining (Zha et al., 1996).

BRK cells expressing ts-p53 (Subramanian et al., 1995) were maintained in DMEM-high glucose 10% serum medium at the nonpermissive temperature of 39°C and cultured at either 5×10^4 cells/0.1 ml in 96-well flat bottom plates or at 10^6 cells/5 ml in 6 cm dishes for cell death assays. The medium was then changed with fresh 32°C medium, and cells were cultured at 32°C with or without various concentrations of oligomycin or antimycin A for 12 hr. The percentage of viable cells was determined by trypan blue dye exclusion, or cell lysates were prepared for immunoblot analysis of Bax expression.

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 (Zha et al., 1996) or either anti-human Bax or anti-mouse/rat Bax rabbit sera (Krajewski et al., 1994; Krajewski et al., 1995).

ATP Measurements

Cellular ATP content was measured as previously reported (Kane et al., 1985) using firefly lantern extract (Luciferase-luciferin, Sigma) with a luminometer. Data were normalized relative to total protein content of cell lysates.

Measurements of Mitochondrial $\Delta\Psi$

Mitochondrial $\Delta\Psi$ was measured using DiOC₆ (Molecular Probes, Inc.) as described (Castedo et al., 1996).

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Bax- and Bak-induced Cell Death in the Fission Yeast *Schizosaccharomyces pombe*

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The effects of the expression of the human Bcl-2 family proteins Bax, Bak, Bcl-2, and Bcl-X_L were examined in the fission yeast *Schizosaccharomyces pombe* and compared with Bax-induced cell death in mammalian cells. Expression of the proapoptotic proteins Bax and Bak conferred a lethal phenotype in this yeast, which was strongly suppressed by coexpression of the anti-apoptotic protein Bcl-X_L. Bcl-2 also partially abrogated Bax-mediated cytotoxicity in *S. pombe*, whereas a mutant of Bcl-2 (Gly145Ala) that fails to heterodimerize with Bax or block apoptosis in mammalian cells was inactive. However, other features distinguished Bax- and Bak-induced death in *S. pombe* from animal cell apoptosis. Electron microscopic analysis of *S. pombe* cells dying in response to Bax or Bak expression demonstrated massive cytosolic vacuolization and multifocal nuclear chromatin condensation, thus distinguishing this form of cell death from the classical morphological features of apoptosis seen in animal cells. Unlike Bax-induced apoptosis in 293 cells that led to the induction of interleukin-1 β -converting enzyme (ICE)/CED-3-like protease activity, Bax- and Bak-induced cell death in *S. pombe* was accompanied neither by internucleosomal DNA fragmentation nor by activation of proteases with specificities similar to the ICE/CED-3 family. In addition, the baculovirus protease inhibitor p35, which is a potent inhibitor of ICE/CED-3 family proteases and a blocker of apoptosis in animal cells, failed to prevent cell death induction by Bax or Bak in fission yeast, whereas p35 inhibited Bax-induced cell death in mammalian cells. Taken together, these findings suggest that Bcl-2 family proteins may retain an evolutionarily conserved ability to regulate cell survival and death but also indicate differences in the downstream events that are activated by overexpression of Bax or Bak in divergent cell types.

INTRODUCTION

Programmed cell death (PCD) plays important roles in tissue homeostasis and developmental elimination of redundant or excess cells in essentially all multicellular organisms (reviewed in Ellis *et al.*, 1991; Ucker, 1991; Williams, 1991; Korsmeyer, 1992; Reed, 1994). In many cases, PCD involves a characteristic set of mor-

phological events, known as apoptosis, which include plasma membrane blebbing, cell shrinkage, chromatin condensation, and nuclear fragmentation, followed ultimately by the budding off of cellular fragments which are cleared by phagocytosis (Wyllie *et al.*, 1980). Apoptosis is also often accompanied by DNA fragmentation, resulting from the activation of endonucleases which initially cleave the genomic DNA at its most assessable locations, between the nucleosomes, thus generating an oligonucleosomal degradation pattern which appears as a "ladder" of DNA bands that can be detected by conventional agarose gel electrophoresis (Wyllie *et al.*, 1984). Many of the morpholog-

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ical features of apoptotic cells may also be the result of specific proteolytic cleavage events mediated at least in part by the interleukin-1 β -converting enzyme (ICE)/CED-3 family of cysteine proteases (reviewed in Martin and Green, 1995). These proteases have specificity for aspartic acid in the P1 position (aspases) and mediate, directly or indirectly, the cleavage of a number of cellular proteins during apoptosis, including those associated with the nuclear matrix (PARP, topoisomerase I), nuclear envelope (lamins, SREBPs), and cytoskeleton (actin, gas-2, fodrin; Ucker *et al.*, 1994; Brancolini *et al.*, 1995; Martin *et al.*, 1995; Tewari *et al.*, 1995; Kayalar *et al.*, 1996; Wang *et al.*, 1996).

One of the distinctive features of PCD and apoptosis is the cell autonomous nature with which these forms of cell death occur, with the dying cells serving as active participants in their own demise (i.e., committing suicide; Ucker, 1991). Cell suicide behaviors have been observed even in some unicellular organisms, raising speculations that the cell death mechanisms currently recognized as apoptosis and PCD in multicellular organisms may have very primitive evolutionary origins (reviewed in Shub, 1994; Yarmolinsky, 1995; Ameisen, 1996). For example, some strains of *Escherichia coli* will activate the expression of genes that trigger cell death when infected with bacteriophages, presumably as a mechanism for limiting viral replication and thus ensuring the survival of the clonal descendants of an infected bacterium. Moreover, many other examples of cell death exist in unicellular bacterial species that could reasonably be classified as suicide or programmed cell death in that the cell is an active participant in its own demise and the death of individual cells affords survival value to other cells that derive from common antecedents, including the cell deaths associated with 1) restriction modification systems involving restriction endonucleases and counteracting methylases; 2) cell suicide following DNA damage induced by irradiation or chemicals in bacteria carrying colicin toxin genes; 3) the death of "mother cells" during sporulation of bacilli species; and 4) the death and subsequent cannibalization of cells during fruiting body formation by myxobacteria (reviewed in Shub, 1994; Yarmolinsky, 1995). In addition, cell death occurring with the classical features of apoptosis has also been reported in some eukaryotic unicellular organisms, such as in *Trypanosoma* species which have been shown to undergo apoptotic cell death in response to high cell densities or insufficient nutrients (Ameisen *et al.*, 1995; Welburn *et al.*, 1996).

Several genes that regulate programmed cell death and apoptosis have been identified in both vertebrate and invertebrate metazoan species, including nematodes, flies, mice, and humans (reviewed in Vaux and Strasser, 1996). The finding of significant sequence homology among some of these cell death-regulatory genes and their encoded proteins has suggested the

existence of an evolutionarily conserved pathway that controls cell survival and death (reviewed in Ellis, 1991; Vaux and Strasser, 1996). This notion is further supported by the discovery of structurally and functionally related genes in several viruses that infect mammalian or insect species (Weiss *et al.*, 1987; Rao *et al.*, 1992; Henderson *et al.*, 1993; Clem and Miller, 1994). Furthermore, the discovery that some PCD-regulatory genes have homologues in both plants and animals (such as the anti-apoptotic gene *dad-1*) suggests that some portions of the cell death machinery may have evolved originally in unicellular organisms, before the divergence of the plant and animal kingdoms (Apte *et al.*, 1995).

Among the more prominent regulators of apoptosis are members of the Bcl-2 family of proteins. These proteins appear to regulate a distal step in what may represent a final common pathway for programmed cell death and apoptosis. Some of the members of this protein family function as suppressors of apoptosis, whereas others act as promoters of cell death (reviewed in Vaux, 1993; Reed, 1994; Nuñez and Clarke, 1994; Oltvai and Korsmeyer, 1994). Interestingly, although best known for its ability to regulate apoptotic cell death, the anti-apoptotic protein Bcl-2 has also been reported to be capable of suppressing necrotic cell death under some circumstances (Kane *et al.*, 1993, 1995; Subramanian *et al.*, 1995; Shimizu *et al.*, 1996), suggesting that some overlap exists in the cell death mechanisms which lead to apoptosis and necrosis and implying that Bcl-2 family proteins can potentially influence both of these types of cellular demise. At present, the mechanism by which Bcl-2 family proteins control cell life and death remains enigmatic, principally because the predicted amino acid sequences of these proteins share no significant similarity with other proteins that have a known biochemical activity. The three-dimensional structure of the Bcl-X_L protein, as determined by nuclear magnetic resonance (NMR) and x-ray crystallographic studies, however, is highly reminiscent of some types of pore-forming bacterial toxins, particularly the B-subunit of diphtheria toxin and *E. coli* colicin-A (Muchmore *et al.*, 1996).

Recent studies indicate that overexpression of Bcl-2 or Bcl-X_L, anti-apoptotic members of this family of proteins, can prevent the proteolytic processing and activation of ICE/CED-3 family proteases such as CPP32 during induction of apoptosis in mammalian cells (Armstrong *et al.*, 1996; Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996; Messmer *et al.*, 1996). Furthermore, genetic analysis of the free-living nematode, *Caenorhabditis elegans*, has revealed a requirement for *ced-9*, the worm homologue of *bcl-2*, for suppression of programmed cell deaths mediated via a CED-3 protease-dependent pathway (Yuan *et al.*, 1993; Hengartner and Horvitz, 1994). Bcl-2 family proteins therefore appear to act at a step at or upstream of the ICE/CED-3 family

proteases. Whatever the biochemical process by which Bcl-2 family proteins modulate cell survival and death, elements of this mechanism appear to be well conserved throughout evolution based on the ability of the human Bcl-2 protein to 1) protect insect cells from virus-induced apoptosis (Alnemri *et al.*, 1992); 2) rescue superoxide dismutase- (*sod*) deficient strains of budding yeast (*Saccharomyces cerevisiae*) from cell death induced by growth under aerobic conditions (Kane *et al.*, 1993); and 3) partially substitute for *ced-9*, the nematode homologue of *bcl-2*, in suppressing cell death during development in *C. elegans* (Hockenbery *et al.*, 1990).

The function of Bcl-2 family proteins appears to be regulated, at least in part, by their interactions with each other through a complex network of homo- and heterodimers (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Yin *et al.*, 1994; Bodrug *et al.*, 1995; Hanada *et al.*, 1995; Sedlak *et al.*, 1995). The anti-apoptotic proteins Bcl-2 and Bcl-X_L, for example, bind to the proapoptotic protein Bax and suppress cell death (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Sedlak *et al.*, 1995). Mutant versions of Bcl-2 and Bcl-X_L have been described that fail to heterodimerize with Bax and also lack anti-apoptotic function (Oltvai *et al.*, 1993; Sedlak *et al.*, 1995). Similarly, the wild-type Bak protein, a proapoptotic member of the Bcl-2 protein family, binds to Bcl-X_L and promotes cell death, whereas mutant versions of Bak that are incapable of heterodimerizing with Bcl-X_L are deficient in promoting apoptosis (Chittenden *et al.*, 1995a). In addition, loss-of-function mutants of the Bcl-2 and Bax proteins have been described which have impaired ability to homodimerize (Hanada *et al.*, 1995; Zha *et al.*, 1996a).

The goal of delineating the molecular mechanisms of Bcl-2 family proteins would be greatly facilitated by being able to assess the function of these apoptosis-regulating proteins in more simple eukaryotes that are easily manipulated in terms of genetic analysis. Recently, it has been shown that the proapoptotic protein Bax confers a lethal phenotype when expressed in the budding yeast *S. cerevisiae* (Sato *et al.*, 1994, 1995; Greenhalf *et al.*, 1996) and is primarily targeted to the mitochondrial membranes (Zha *et al.*, 1996b). Analogous to studies performed in mammalian cells, anti-apoptotic members of the Bcl-2 protein family, including Bcl-2, Bcl-X_L, and Mcl-1, suppress the lethal function of Bax in yeast, whereas the proapoptotic protein Bcl-X_S and various deletion mutants of Bcl-2 which are nonfunctional in mammalian cells do not (Sato *et al.*, 1994; Bodrug *et al.*, 1995; Hanada *et al.*, 1995; Greenhalf *et al.*, 1996). Interestingly, even some mutants of Bcl-2 which retain the ability to bind Bax but which are deficient in anti-apoptotic activity in mammalian cells are likewise unable to suppress Bax-mediated lethality in budding yeast (Hanada *et al.*, 1995), implying that the mere binding of anti-apop-

otic Bcl-2 family proteins to Bax can be insufficient to abrogate its function in yeast.

These parallels between the effects of wild-type and mutant Bcl-2 family proteins in budding yeast on cell death suggest that some aspects of the functions of these proteins may be translatable into less complex organisms. Although few details are known concerning the mechanisms by which Bax kills and Bcl-2 protects in budding yeast, preliminary explorations of this topic suggest that it does not involve apoptosis (Nuñez and Clarke, 1994). Since aspects of the cell division mechanisms utilized by fission yeast more closely resemble what occurs in mammalian cells (Alfa *et al.*, 1993), we explored the function of the Bcl-2 family proteins Bax, Bak, Bcl-2, and Bcl-X_L in *S. pombe*. In addition, we extended previous studies of Bcl-2 family protein function in yeast by exploring the morphological characteristics of *S. pombe* induced to die by Bax and its closely related homologue Bak, and asked whether Bax and Bak induce the production of ICE-/CED3-like protease activities in this unicellular organism. Finally, we established that Bax expression induces activation of apoptosis-associated ICE/CED3-like protease activity in mammalian cells and compared this response to that seen in *S. pombe*.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. pombe* strain SOP444 (*h⁺ leu1-32 ura4-D18 his7-366 ade6-M210*) was used for all experiments. Cells were maintained in YEL/YES media before transformation and in EMM, lacking the selective amino acids, after transformation (Alfa *et al.*, 1993).

Cloning of Human Bak cDNA

The human *bak* cDNA was cloned by polymerase chain reaction (PCR) amplification from a HepG2 cDNA library using the primers 5'-ATTCTGGAACTGGGCTC-3' and 5'-TGGAGTGCACCACTTGCTAAAG-3'. The resulting PCR product was digested with *Bam*HI and *Hind*III and cloned into the corresponding sites in Bluescript pSK-II (Stratagene, La Jolla, CA).

Plasmid Constructions

The vectors pREP3X (*leu2* marker) and pREP4X (*ura4* marker), containing the thiamine repressible *umt* promoter, were used for conditional expression of cDNAs in *S. pombe* (Forsburg, 1993). All cDNAs were blunted and subcloned into the *Sma*I site of these vectors. The human *bcl-2* cDNA was excised from pRcCMV-Bcl-2 by digestion with *Xba*I and *Hind*III. The human Bax cDNA was obtained from pcDNA3-*hubax* by digestion with *Eco*RI. The *bcl-X_L* cDNA was taken from pSKIIBcl-X_L by *Eco*RI digestion. The human *bak* cDNA was excised from pSKIIBak by digestion with *Bam*HI and *Hind*III. The p35 cDNA was excised from pPRM-3K-ORF (Sugimoto *et al.*, 1994) by digestion with *Bam*HI. The *bcl-2* (G145A) mutant was constructed by PCR amplification using the plasmid M1-3 (Yin *et al.*, 1994) as a template and the following primers: 5'-ATCAGTCTC-GAGACTATGGCGCAGCTGGGAGA-3' and 5'-ATCGATCTC-GAGTCACTTGTTGGCTCAGATAGGC-3'. The resulting PCR product was digested with *Xho*I and subcloned into pREP3X. The proper construction of all plasmids was confirmed by DNA sequencing.

Transformation and Induction of Protein Expression

Transformations were performed using the lithium acetate method (Moreno *et al.*, 1991). Cells were maintained in media (EMM lacking uracil and/or leucine) containing 5 $\mu\text{g}/\text{ml}$ thiamine to prevent induction of the *nmf* promoter. Cells were then cultured either on plates or in liquid media in the presence or absence of thiamine. Cells in liquid culture were periodically diluted to sustain log phase growth.

Immunoblot Assays

Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in Laemmli buffer. The resulting lysates were kept at -70°C until used, boiled for 15 min, and briefly centrifuged, and the supernatants were run in 12% polyacrylamide gels. Samples were normalized for cell number, determined by optical density at OD_{600} . Immunoblotting was performed using an enhanced chemiluminescence method (Krajewski *et al.*, 1996b). Antibodies used for these studies included polyclonal rabbit antisera huBax 1701, huBak 1764, and huBcl-X 1695 and the anti-human Bcl-2 monoclonal antibody 4D7 (Krajewski *et al.*, 1994–1996a; Reed *et al.*, 1992).

Electron Microscopy (EM)

Cells were harvested at the indicated times by centrifugation, washed once with phosphate-buffered saline, and fixed for 30 min in 4 M phosphate buffer containing 3% glutaraldehyde. After two washing steps with 4 M phosphate buffer, cell pellets were embedded in Epon. Cells were postfixed and counterstained with 0.5% osmium tetroxide and 1% uranyl acetate, cut ultrathin, and placed on grids (Krajewski *et al.*, 1993). Sections were imaged using a Hitachi H-600 electron microscope.

Cysteine Protease Enzyme Assays

Lysates (50 μg total protein) were prepared from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 transformants by glass bead disruption, adjusted to 150 μl total volume in buffer A [20 mM *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, 10% sucrose], and added to a single well of a 96-well plate. Reactions were started by addition of 50 μl of fluorogenic tetrapeptide-aminomethylcoumarin (AMC) substrates to a final concentration of 10 μM . The following substrates were used: DEVD-AMC and YVAD-AMC. Fluorescent AMC product formation was measured at excitation 360 nm, emission 460 nm, using a cytofluor II fluorescent plate reader (Millipore, Bradford, MA). The change in fluorescence was measured over 1 h and expressed as the change in fluorescence per hour. 293 cell lysates were prepared in buffer A from 1.8×10^6 cells on 10-cm dishes 24 h after transfection using standard calcium phosphate methods with either 50 μg pCIneo (Promega, Madison, WI) alone, 50 μg total of pCIneo/pCIP35, pCIBax/pCIneo, or pCIBax/pCIP35 at equal molar ratios. Fifty micrograms of extract were used per assay point and determinations were made in duplicate.

p35 Inhibition Assays

Lysates (50 μg total protein) from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 double transformants or pRep transformants as a control were prepared by glass bead disruption or, alternatively, 50 ng of recombinant 6His-p35 protein were used. Yeast cell lysates or recombinant p35 were adjusted to a total volume of 100 μl in buffer A, added to a single well of a 96-well plate, and 1 U of recombinant purified active CPP32 protease (Nicholson *et al.*, 1995) was added in a volume of 75 μl . The substrate DEVD-AMC was then added in 25 μl of buffer A to a final concentration of 10 μM and release of the fluorogenic AMC product was monitored as described above. Inhi-

bition of CPP32 activity by lysates or recombinant purified p35 protein was expressed as a percentage relative to the DEVD-AMC cleaving activity obtained when 1 U of CPP32 is combined with 75 μg of Rep3X control extract.

Mammalian Cell Viability Assay

293 cells (2×10^5 /well) in 6-well plates were transfected by calcium phosphate precipitation with pCIneo/pCIBax/pCIP35 as appropriate in an equal molar ratio. One-fifth the amount of pRc π V- β -galactosidase expression vector was cotransfected as a marker for transfected cells. Twenty-four hours after transfection, cells were fixed and LacZ-expressing cells were visualized using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside as a substrate. Percentage of death was determined as the ratio of round blue cells to total (round + flat) blue cells $\times 100$. Experiments were done in triplicate and are expressed as the mean value $\pm$ SD.

RESULTS

Expression of Bax or Bak Confers a Lethal Phenotype in *S. pombe*

cDNAs encoding the human Bax and Bak proteins were subcloned into the *S. pombe* vector pREP3X, which utilizes an *nmf* ("no message with thiamine") promoter for conditional expression of heterologous cDNAs. The presence of thiamine in the growth media represses expression of this promoter, whereas removal of thiamine induces the promoter with a lag time of ~ 12 h (Maundrell, 1990). *S. pombe* cells (strain SOP444) were transformed with the Bax and Bak expression plasmids or the parental pREP3X plasmid as a control. Transformants were initially plated on media containing thiamine, thus repressing the *nmf* promoter, and then single colonies were isolated, streaked onto plates containing or lacking thiamine, and assayed for their ability to grow. As shown in Figure 1A, yeast calls carrying the Bax expression plasmid were markedly impaired in their ability to grow on plates lacking thiamine compared with cells containing the control plasmid. In contrast, transformants containing the Bax and control plasmids grew equally well on thiamine-supplemented medium. Similar results were obtained for Bak (our unpublished results).

To determine whether Bax and Bak induce cell death as opposed to a reversible growth arrest, yeast cells that had been transformed with the Bax, Bak, or control plasmids were cultured in liquid media without thiamine to induce the *nmf* promoter. Aliquots of these cultures were then removed at various times and plated onto solid media containing thiamine. If the growth inhibition mediated by Bax and Bak was reversible, then plating the cells on thiamine-containing medium would be expected to rescue the cells and result in colony-forming units at frequencies comparable to cells carrying the control plasmid. Conversely, if Bax and Bak kill *S. pombe*, then the cells should not be rescuable on thiamine-supplemented medium.

As shown in Figure 2, A and B, culturing cells that contained Bax or Bak expression plasmids in thiamine-deficient medium (to induce the *nmf* promoter) resulted in a time-dependent decline in the numbers of viable clonogenic cells that could be subsequently recovered on plates containing thiamine, with >75% inhibition of viable colony formation within ~10 h and nearly complete suppression of colony formation occurring within ~14 h (data normalized relative to efficiency of rescue of cells containing the pREP parental plasmid as a control). Using trypan blue staining, we observed the appearance of dying cells around 10–14 h (our unpublished results), consistent with the plating results. These data are consistent with a lethal effect of Bax and Bak on *S. pombe*.

Bcl-2 and Bcl-X_L Rescue *S. pombe* from Lethal Effects of Bax and Bak

Bcl-2 and Bcl-X_L are anti-apoptotic members of the Bcl-2 protein family that have been reported to suppress the cell death-promoting effects of Bax and Bak in mammalian cells (Oltvai *et al.*, 1993; Chittenden *et al.*, 1995a,b; Farrow *et al.*, 1995). Bcl-X_L can heterodimerize with both Bax and Bak, whereas Bcl-2 interacts efficiently with Bax but more poorly with Bak (Farrow *et al.*, 1995). Certain loss-of-function mutants of Bcl-2, such as Bcl-2 (G145A), fail to heterodimerize with Bax and also do not suppress apoptosis (Yin *et al.*, 1994). To determine whether the expression of Bcl-2 or

Bcl-X_L can rescue fission yeast from Bax- or Bak-induced cell death, *S. pombe* cells containing Bax or Bak expression plasmids were transformed with expression plasmids encoding wild-type Bcl-2, mutant Bcl-2 (G145A), or Bcl-X_L under the control of the *nmf* promoter in pREP4X which carries a URA4 selectable marker as opposed to the LEU2 marker in pREP3X plasmid from which Bax and Bak were expressed.

After initial plating on thiamine-containing medium to repress the *nmf* promoter and thus prevent expression of Bax and Bak, single colonies of these transformants were streaked onto plates that contained or lacked thiamine. Both Bcl-2 and Bcl-X_L restored growth to Bax- and Bak-expressing cells when plated on thiamine-deficient medium, as illustrated by the representative experiment shown in Figure 1B. In contrast, Bax- and Bak-expressing cells that contained the plasmid encoding the mutant Bcl-2 (G145A) protein failed to grow when streaked on thiamine-deficient medium. Comparable growth was observed for all transformants when streaked onto plates containing thiamine-supplemented medium, demonstrating the specificity of these results (Figure 1B). Bcl-X_L consistently afforded more protection than Bcl-2 in these assays, not unlike some results involving mammalian cells (Gottschalk *et al.*, 1994). Similar results were obtained with ~10 other independent clones of each transformation.

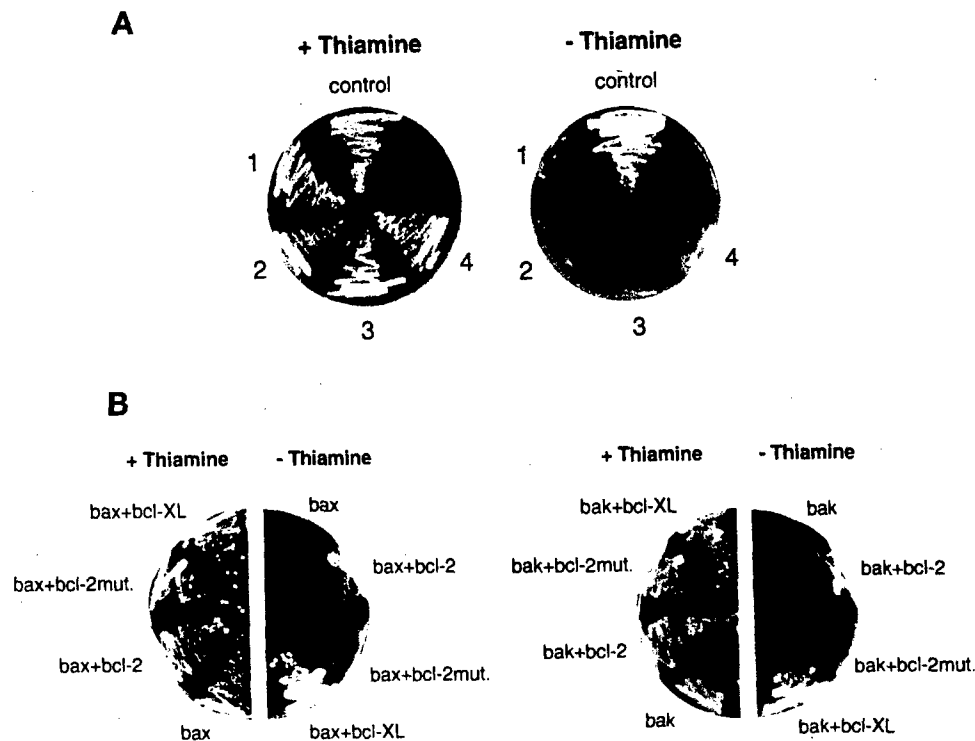


Figure 1. Bax/Bak-induced cell death in *S. pombe* and rescue by Bcl-2 and Bcl-X_L. In A, *S. pombe* cells were transformed with a Bax expression plasmid. Four independent clones were tested on selective media with and without thiamine (1–4). In the absence of thiamine Bax is expressed. Controls were transformed with the parent plasmid lacking a *bax* cDNA insert. In B, individual colonies of *S. pombe* transformants containing pREP3X-Bax or pREP3X-Bak with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or REP4X-Bcl-X_L plasmids were streaked onto plates containing or lacking thiamine and growth was monitored 4 d later.

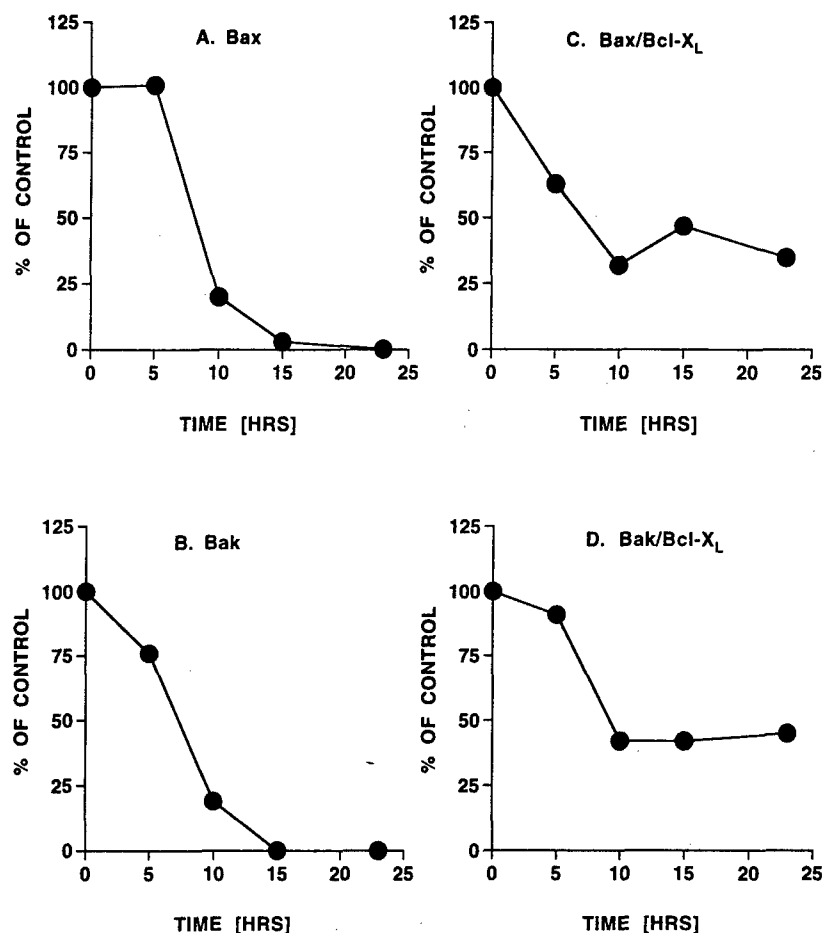


Figure 2. Bax- and Bak-mediated growth inhibition is irreversible. Cells were transformed with pREP3X and pREP4X plasmids either lacking a cDNA insert (parental) or containing *bax*, *bak*, or *bcl-X_L* cDNAs and grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *nmf* promoter. At various times thereafter, cells were plated onto thiamine-containing solid medium, and the relative numbers of colonies were determined based on comparisons with cells cotransformed with the pREP3X and pREP4X parental plasmids. (A) Bax; (B) Bak; (C) Bax and Bcl-X_L; (D) Bak and Bcl-X_L.

The ability of Bcl-X_L and Bcl-2 to rescue Bax- and Bak-expressing *S. pombe* cells was also observed in experiments where cells were transiently cultured in thiamine-deficient medium to induce the *nmf* promoter in the pREP3X and pREP4X plasmids that contained *bax*, *bak*, *bcl-2*, and *bcl-X_L* cDNAs, and then at various times thereafter were replated onto thiamine-containing medium to shutoff expression of Bax and Bak. As shown in Figure 2, 25–50% of the cells that had been cotransformed with the pREP4X-Bcl-X_L plasmid and either the pREP3X-Bax (Figure 2C) or the pREP3X-Bak (Figure 2D) plasmid were rescuable when plated on thiamine-containing plates after a period of growth in thiamine-deficient medium. Similar results were obtained with Bcl-2, but the rescue was less effective compared with Bcl-X_L (our unpublished results). In contrast, the cells transformed with the pREP4X parental plasmid (lacking a *bcl-X_L* or *bcl-2* cDNA) and either pREP3X-Bax or pREP3X-Bak were all unrecoverable within 24 h of growth in thiamine-deficient medium, as mentioned above (Figure 2, A and B). Since coexpression of Bcl-X_L allowed cells to survive a transient exposure to Bax or Bak, resulting in

increased numbers of viable clonogenic cells, we conclude that Bcl-X_L is capable of abrogating the lethal effects of Bax and Bak in *S. pombe*.

Finally, the effects of Bcl-2 and Bcl-X_L on Bax- and Bak-expressing *S. pombe* cells were examined by growing cells in thiamine-deficient liquid culture medium and spectrophotometrically assessing the cell culture densities over a ~2-day period by measuring the absorbance at 600 nm. As shown in Figure 3, when *S. pombe* cells containing the pREP3X-Bax or pREP3X-Bak plasmids in combination with the pREP4X control plasmid lacking a *bcl-2* or *bcl-X_L* cDNA were switched from thiamine-containing medium to thiamine-free medium, growth as defined by OD_{600 nm} began to cease by ~24 h. In contrast, the cell densities in cultures of *S. pombe* cells that contained the pREP4X-Bcl-X_L or pREP4X-Bcl-2 expression plasmids along with either pREP3X-Bax or pREP3X-Bak continued to increase at 24 h, with faster rates of growth seen in the Bcl-X_L- than the Bcl-2-expressing cells (Figure 3, A and B). Cells that coexpressed the Bcl-2 (G145A) mutant protein with either Bax or Bak, on the other hand, ceased growing after ~24 h, thus verifying the speci-

ficity of the results. Growth of all transformants was comparable in thiamine-supplemented medium (our unpublished results), consistent with the thiamine-mediated repression of the *nmt* promoter.

As an additional control, we examined the effects of Bcl-2 and Bcl-X_L on the growth of *S. pombe* in the absence of Bax or Bak. Cells transformed with the pREP4X-Bcl-2 plasmid and pREP3X, the same parental plasmid from which Bax and Bak were expressed but without the *bax* or *bak* cDNAs, grew at rates comparable to control transformants containing only the pREP4X and pREP3X parental plasmids (Figure 3C). Thus, Bcl-2 by itself did not have an effect on growth. In contrast, yeast cells transformed with pREP4X-Bcl-X_L and pREP3X grew somewhat faster than control transformants containing the pREP4X and pREP3X parental plasmids in some experiments (e.g., Figure 3), but grew at rates essentially identical to control transformants in others. Taken together, these data in Figure 3 support the contention that Bcl-2 and Bcl-X_L specifically suppress the lethal effects of Bax and Bak in *S. pombe*, whereas the Bcl-2 (G145A) mutant does not.

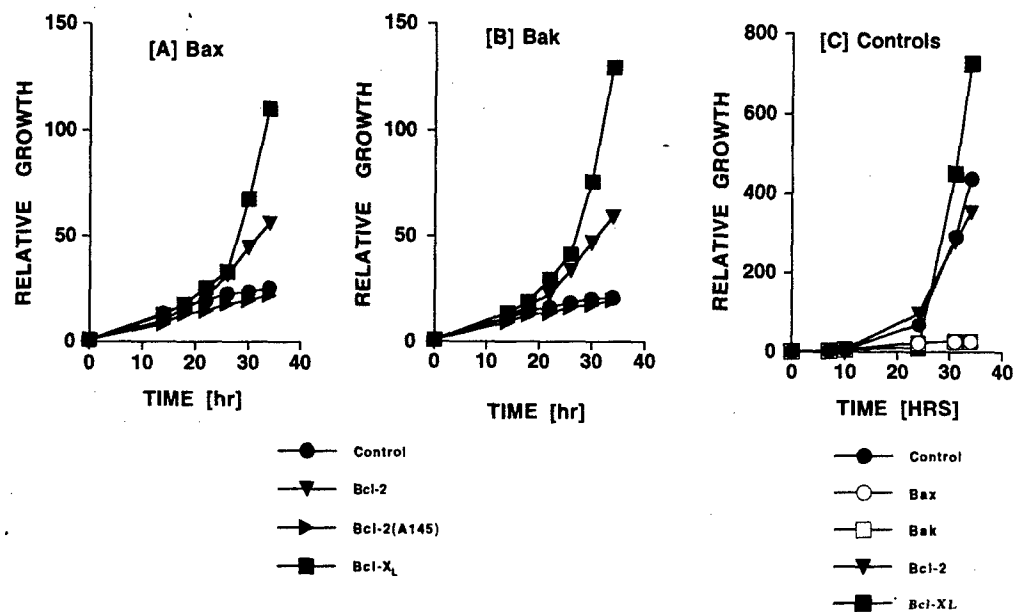
Time Courses of Bak and Bax Protein Accumulation Correlate with Kinetics of Growth Inhibition in *S. pombe*

The time courses of Bax, Bak, Bcl-X_L, and Bcl-2 protein accumulation were evaluated by immunoblotting of

the various *S. pombe* transformants after inducing cells in thiamine-deficient medium. These experiments were performed in parallel with the growth studies described above in Figure 3, thus allowing direct comparisons of expression of these Bcl-2 family proteins with function. As shown in Figure 4, production of the Bax and Bak proteins was either undetectable or just barely detectable by immunoblotting at the earliest time examined (14 h) but became maximal at 24–36 h after seeding cells into thiamine-deficient medium. When compared with the data above in Figure 3, these immunoblotting data therefore suggest that accumulation of the Bax and Bak proteins coincides roughly with the onset of growth inhibition. The observation that 10–15 h of Bax or Bak induction renders cells nonviable (Figure 2) suggests that these proteins can have effects on *S. pombe* cells at modest expression levels.

The time courses of induction of Bcl-2 and Bcl-X_L were similar to those of Bax and Bak, as might be expected given that the same *nmt* promoter was used for driving expression of these anti-apoptotic proteins in *S. pombe*. Note that the wild-type and mutant Bcl-2 (G145A) proteins were produced at comparable levels in yeast, thus excluding insufficient production of the mutant Bcl-2 (G145A) protein as an explanation for its failure to rescue cells from Bax- and Bak-induced death. The levels of Bax and Bak produced in yeast containing the Bcl-X_L or Bcl-2 expression plasmids

Figure 3. Time course of effects of Bax and Bak on growth of *S. pombe*. Cells transformed with pREP3X plasmids containing *bax* or *bak* cDNAs with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or pREP4X-Bcl-X_L plasmids were grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *nmt* promoter. At various times thereafter, relative cell growth was estimated by OD_{600 nm}. Cells were maintained in mid-log phase growth throughout the experiment by dilution in fresh thiamine-deficient medium prior to the OD_{600 nm} reaching 0.7. Data represent calculated theoretical total ODs. C (control) represents cells cotransformed with the pREP3X and pREP4X control



plasmids. In A, all transformants contained the pREP3X-Bax plasmid along with various pREP4X plasmids as indicated. In B, all transformants contained the pREP3X-Bak plasmid along with other plasmids. In C, cells cotransformed with pREP3X parental plasmid and either pREP4X parental plasmid (C, ●), pREP4X-Bcl-2 (○), or pREP4X-Bcl-X_L (■). Alternatively, cells were transformed with the pREP4X parental plasmid and either pREP3X-Bax (□) or pREP3X-Bak (□).

were typically comparable to or even greater than those seen in cells that expressed Bax or Bak in the absence of these anti-apoptotic proteins, thus discounting lower levels of Bax and Bak protein as the explanation for the rescue of *S. pombe* by Bcl-X_L and Bcl-2 (our unpublished results). Moreover, the maximum relative levels of Bax and Bak reached in fission yeast cells were comparable to those seen in several mammalian tissues when samples were normalized for total protein content (Krajewski *et al.*, 1996a, and our unpublished results). Thus, it cannot be argued that supraphysiological levels of these cell death-promoting proteins were obtained in *S. pombe*. Analysis of control transfected *S. pombe* cells that contained the same pREP 3X and pREP 4X plasmids without *bax*, *bak*, *bcl-X_L*, or *bcl-2* cDNA inserts ("C") revealed no proteins that could be detected by antibodies directed against the human proteins, thus confirming the specificity of the immunoblotting results (Figure 4).

Morphological Analysis of Bax- and Bak-expressing *S. pombe* by EM

The distinct morphological features that characterize apoptosis in animal cells are best demonstrated by

transmission EM (reviewed in Wyllie *et al.*, 1980). We therefore analyzed by EM the morphology of *S. pombe* cells undergoing cell death as a result of expressing Bax or Bak. As shown in Figure 5, striking differences were seen in the morphology of control cells that carried plasmids lacking cDNA inserts (A) and the Bax-expressing *S. pombe* cells (B). Bax-expressing cells uniformly developed massive vacuolization of the cytosol. The cytosol also became electron dense ("cytosolic condensation"), similar to previous descriptions of programmed cell death in plant and animal cells. However, the size distribution of control and dying Bax-expressing cells was approximately the same, and thus Bax expression did not induce the cell shrinkage typical of mammalian cell apoptosis. Also, unlike apoptosis in animal cells, plasma membrane blebbing was not observed, but at higher resolution (our unpublished results) invaginations of the plasma membrane were commonly present beneath the cell wall in Bax- and Bak-expressing cells. Foci of chromatin condensation were present in the nucleus of Bax-expressing cells but not in the control cells. No evidence of nuclear fragmentation or of chromatin margination against the nuclear envelope was obtained by EM

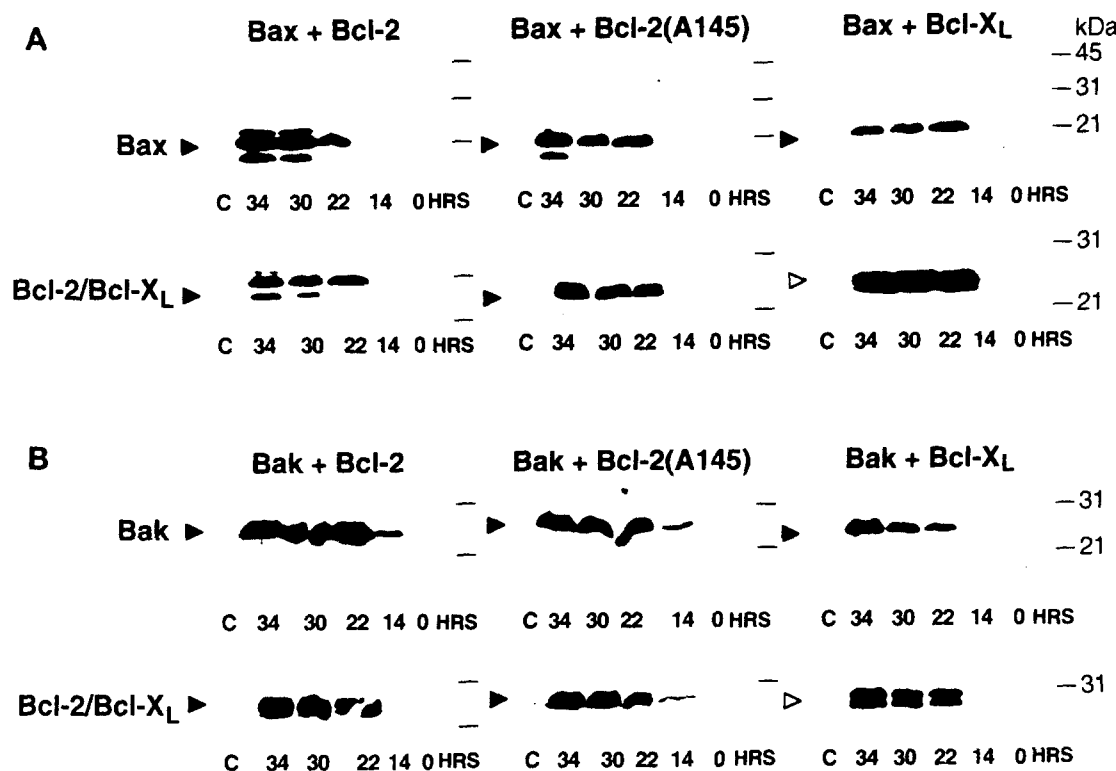


Figure 4. Immunoblot analysis of Bcl-2 family proteins in *S. pombe*. Cells that had been cotransformed with pREP3X plasmids encoding either Bax or Bak and pREP4X plasmids encoding Bcl-2, Bcl-2 (G145A), or Bcl-X_L were grown as described in Figure 3, and aliquots of the cells were removed, lysed, and their proteins analyzed by SDS-PAGE/immunoblotting using antisera specific for the human Bax, Bak, Bcl-2, and Bcl-X proteins (Krajewski *et al.*, 1996b). Detection was accomplished with an enhanced chemiluminescence method using a horseradish peroxidase-conjugated secondary goat anti-rabbit antibody as described (Krajewski *et al.*, 1996b). C represents control cells cotransformed with pREP3X and pREP4X parental plasmids. In the rows labeled as Bcl-2/Bcl-X_L, the closed arrows indicate the positions of the Bcl-2 protein, whereas the open arrows indicate the position of the Bcl-X_L protein. The Bcl-X_L protein routinely migrates as a doublet in SDS-PAGE (Krajewski *et al.*, 1994). The smaller band seen in some cases for Bcl-2 probably represents a partial degradation product.

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analysis. Similar results were obtained for Bak-expressing *S. pombe* cells (our unpublished results). Despite the presence of focal chromatin condensation in Bax- and Bak-expressing *S. pombe* cells, we were unable to detect the presence of fragmented DNA having the oligonucleosomal pattern typical of apoptotic cells by agarose gel electrophoresis.

To explore whether these morphological changes were specifically associated with the cell death process induced by Bax and Bak versus merely a characteristic of producing Bax or Bak protein in this fission yeast, EM analysis was performed for *S. pombe* cells coexpressing Bcl-X_L with either Bax or Bak. Immunoblot analysis confirmed that the relative levels of the Bax and Bak proteins produced in these Bcl-X_L-expressing cells were as high as those seen in cells expressing Bax or Bak alone without Bcl-X_L (our unpublished results). In fact, the levels of Bax and Bak tended to be approximately twofold to threefold higher in the Bcl-X_L-expressing cells (our unpublished results), possibly because these cells were able to tolerate higher levels of these proapoptotic proteins. EM analysis of these cells revealed that about one-half to three-quarters of the Bcl-X_L-expressing cells retained the morphological features of control cells, whereas the others developed the cytosolic condensation, vacuolization, and focal chromatin condensation that was seen in essentially all of the cells that expressed Bax or Bak alone in the absence of Bcl-X_L (Figure 5C). These morphological data are in general accord with the clonogenic assay (Figure 2) which also demonstrates a partial rescue from Bax/Bak-induced death. The incomplete rescue in these assays is possibly due to clonal differences in the copy number of the episomal plasmid from which Bcl-X_L was expressed.

These morphological features of dying *S. pombe* cells were not unique to Bax and Bak, since overexpression of the protein tyrosine phosphatase pyp1 produced many of the same changes but with a delay of ~1 d

relative to Bax and Bak. The pyp1 phosphatase induces a G₂-M arrest followed by cell death (Ottillie *et al.*, 1992). EM analysis of pyp1-expressing cells demonstrated elongation of the cells consistent with a G₂-M arrest (our unpublished results), followed ~1 d later by the massive vacuolization and other changes seen in Bax- and Bak-expressing *S. pombe* cells. Multifocal chromatin condensation, however, was less evident in these pyp1-expressing cells compared with Bax and Bak (our unpublished results). Similar morphological features have been described recently for *S. cerevisiae* overexpressing histone H1 (Miloshev *et al.*, 1994), further suggesting that the morphology produced as a result of ectopic expression of Bax and Bak in *S. pombe* is not unique to these mammalian proapoptotic proteins.

Absence of Detectable ICE/CED-3-like Protease Activity in *S. pombe* Cells Undergoing Bax- and Bak-mediated Cell Death

The induction of apoptosis in mammalian cells is typically accompanied by the activation of proteases of the ICE/CED-3 family (reviewed in Martin and Green, 1995). These proteases cleave their substrates specifically after aspartic acid. To address the question of whether *S. pombe* cells undergoing cell death due to the expression of *bax* contain similar protease activities, lysates were prepared from cells after inducing Bax expression from the *nmt* promoter in pREP3X as well as from control cells containing the same plasmid without Bax. In vitro assays for ICE/CED-3 protease activity were then performed using two different fluorogenic peptide substrates that are known to be effective for one or more ICE/CED-3 family proteases: YVAD-AMC and DEVD-AMC (Nicholson *et al.*, 1995). Neither of these substrates was cleaved by *S. pombe* lysates expressing Bax (Figure 6A). In contrast, Bax

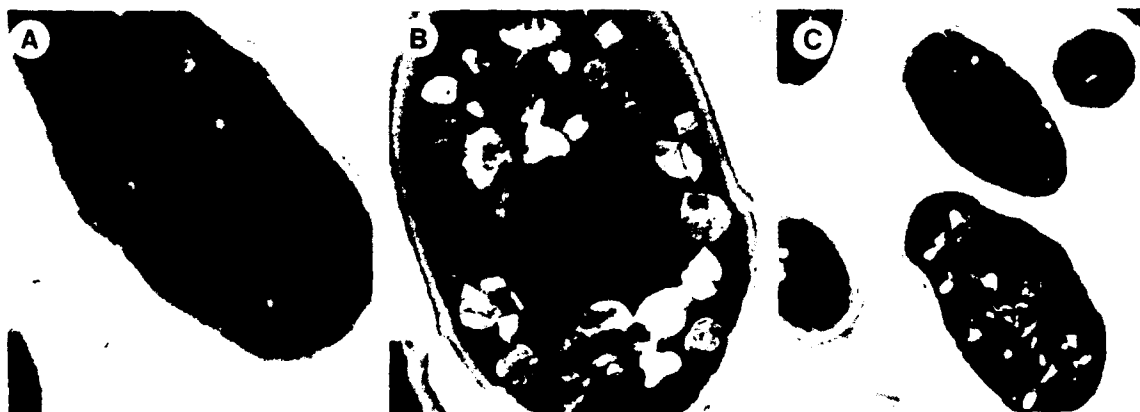


Figure 5. EM analysis of Bax-expressing *S. pombe*. Cells were transformed with either pREP3X (A), pREP3X-Bax (B), or pREP3X-Bax plus pREP4X-Bcl-X_L (C) and then grown for 1 d in thiamine-deficient medium prior to fixing cells and performing EM analysis.

overexpression in mammalian 293 cells leads to an increase in DEVD-AMC cleaving activity (Figure 6B).

p35 Does not Rescue Bax-induced Cell Death in S. pombe

To further explore the possibility that ICE/CED-3-like proteases are involved in the Bax/Bak-mediated cell death process in *S. pombe*, we constructed an expression plasmid containing a cDNA for the baculovirus p35 gene under the control of the *nmt* promoter. The p35 protein binds to and inhibits the enzymatic activities of all known ICE/CED-3 family proteases (Rabizadeh *et al.*, 1993; Hay *et al.*, 1994; Sugimoto *et al.*, 1994; Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). The p35 protein has also been shown to block apoptosis and programmed cell death in a wide range of animal species or cells derived from them, including nematodes, flies, ants, mice, rats, and humans (Clem and Miller, 1994; Pronk *et al.*, 1996; White *et al.*, 1996). Yeast containing the pREP3X-Bax, pREP3X-Bak, or pREP3X plasmids were transformed with pREP4X-p35 or the same vector without a cDNA insert as a control. Cells were then grown to mid-log phase in thiamine-deficient medium to induce the expression of p35 and Bax and Bak. Cell lysates were prepared and tested for their ability to inhibit the activity of the ICE/CED-3-family protease CPP32 using an *in vitro* protease assay. These experiments demonstrated that an inhibitory activity consistent with p35 was specifically produced in the cells transformed with the p35 but not in cells transformed with the parental plasmid pRep (lysates normalized for total protein content). As an additional control, recombinant purified p35 protein (2.5 ng) was added to this *in vitro* protease assay, resulting in ~99% inhibition of CPP32 activity (Figure 6C).

Despite the production of biologically active p35 in yeast, no suppression of the lethal phenotype conferred by Bax and Bak was observed in cells that coexpressed p35 with these proapoptotic proteins (our unpublished results). Similarly, Bax- and Bak-induced lethality were also not impaired in yeast in which p35 expression was driven by a constitutive ADH promoter (our unpublished results). In contrast, in 293 cells both the Bax-induced DEVD-AMC cleaving activity (Figure 6B) and Bax-induced cell death are inhibited by overexpression of p35 (Figure 6D). Taken together, these results suggest that *S. pombe* lack protease activities similar to the ICE/CED-3 family proteases that become activated during apoptosis in animal cells. Furthermore, such proteases are evidently not the effectors of cell death induced by Bax and Bak in fission yeast.

DISCUSSION

The purpose of this study was to investigate the effects of Bax and Bak expression in the fission yeast *S. pombe* and to compare it to Bax-induced cell death in mammalian cells. Several observations demonstrate similarities between the effects of these proteins in fission yeast and mammalian cells. First, Bax and Bak expression resulted in cell death in yeast. Moreover, the effects of Bax and Bak were not related simply to an arrest of cell growth, but rather were due to cell death, as determined by clonogenic assays. Second, the lethal effect of Bax and Bak was specifically abrogated by coexpressing the anti-apoptotic proteins Bcl-X_L and Bcl-2, but not a mutant of Bcl-2 that fails to suppress the proapoptotic effects of Bax in animal cells (Yin *et al.*, 1994). These observations therefore are similar to reports involving mammalian cells, where the effects of these Bcl-2 family proteins on cell survival and death have been studied previously. Other observations, however, demonstrated differences between yeast and mammalian cells with regard to the cell death phenotype conferred by Bax and Bak. First, the morphology of *S. pombe* cells undergoing Bax/Bak-induced cell death involved cytoplasmic vacuolization, cytosolic condensation, and multifocal nuclear condensation, but not the cell shrinkage, nuclear fragmentation, and chromatin margination characteristic of apoptosis. Second, internucleosomal DNA fragmentation was not observed in this fission yeast, but is often found in mammalian cells undergoing apoptosis. Third, no ICE/CED-3-like protease activities were detected in yeast induced to express Bax or Bak. Fourth, the protease inhibitor p35 failed to prevent Bax/Bak-induced cell death in *S. pombe*.

The cell death process mediated by Bax and Bak in fission yeast involved morphological changes that were not consistent with apoptosis as defined in mammalian cells, but instead was more reminiscent of programmed cell death in plants and protists. In particular, the striking cytoplasmic vacuolization, cytosolic condensation, and multifocal nuclear condensation seen in *S. pombe* when induced to die by Bax and Bak were highly similar to the programmed cell death reported previously for the slime mold *Dictyostelium* (Cornillon *et al.*, 1994). Like the death of stalk cells seen in the multicellular structures formed by *Dictyostelium* during times of nutritional insufficiency, the induction of cell death by Bax and Bak in *S. pombe* did not involve DNA fragmentation in the oligonucleosomal pattern that is typical of apoptosis in many types of animal cells. Since pulse-field gel electrophoretic analysis of yeast chromosomal DNA was not performed, we cannot exclude the possibility that DNA fragmentation into higher molecular weight fragments occurred, as has been reported for some types of mam-

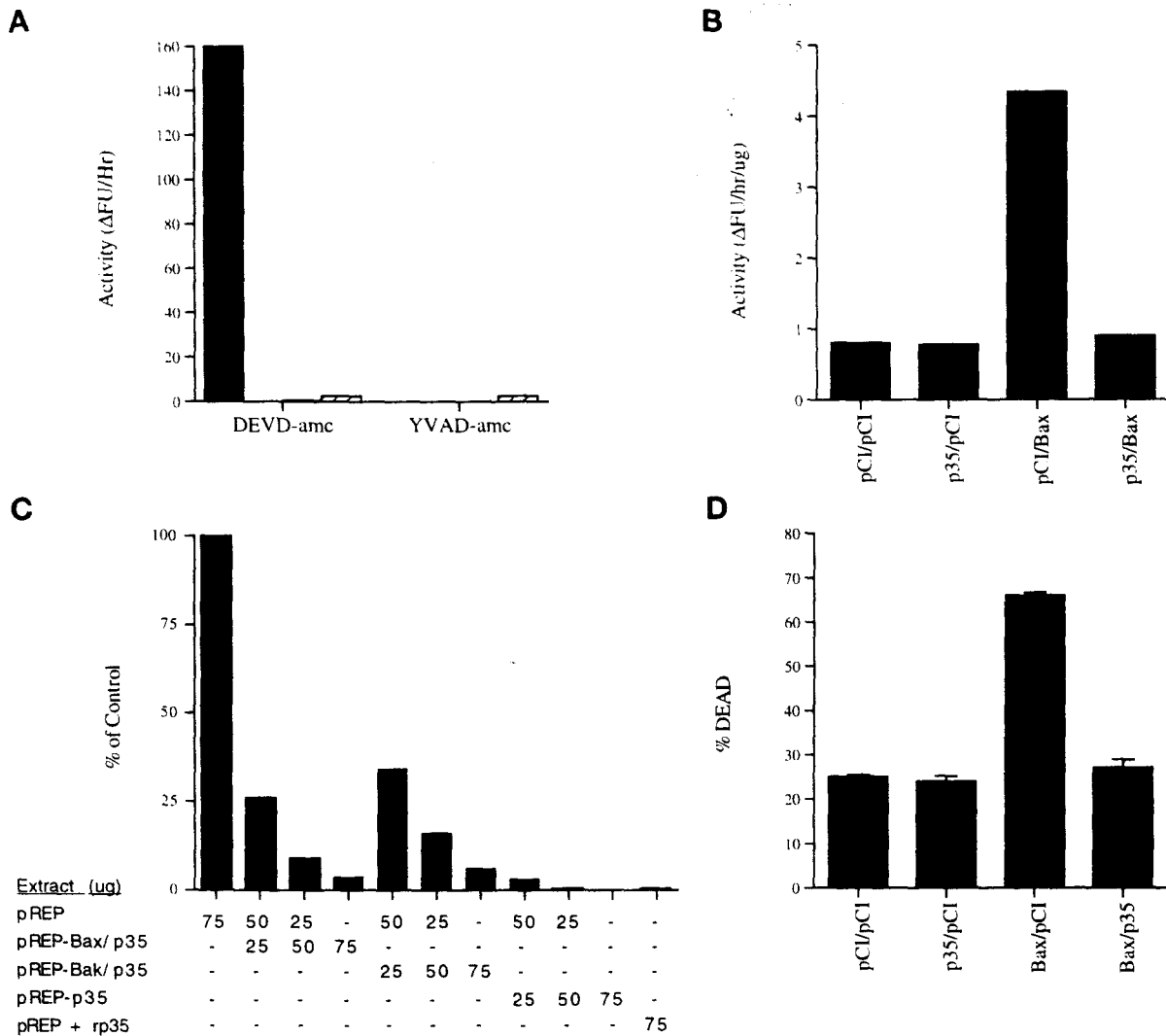


Figure 6. Lack of ICE/CED-3-like protease activity in Bax-transformed *S. pombe* cells. In A, lysates were prepared from *S. pombe* cells cotransformed with pREP4X and either the pREP3X parental plasmid (hatched bar; no activity) or pREP3X-Bax (stippled bar) at 12 h after diluting them into thiamine-deficient medium. The relative amount of release of fluorogenic product (AMC) from the peptide substrates DEVD-AMC and YVAD-AMC in 1 h is shown in arbitrary fluorescence units (Δ FLU). As a control, 1 U of purified recombinant CPP32 (solid bar) is shown using DEVD-AMC as a substrate. (This protease cleaves the peptide substrate DEVD-AMC (Nicholson *et al.*, 1995).) In B, 293 lysates were prepared 21 h after transfection with 50 μ g of pCIneo or 25 μ g each of pCIneo/p35, Bax/pCIneo, or Bax/p35. The relative amount of release of fluorogenic product (AMC) from peptide substrate (DEVD-AMC) (Δ FLU) in 1 h is shown in arbitrary fluorescence units. In C, *S. pombe* cells were transformed with either pREP3X-Bax, pREP3X-Bak, or pREP3X parental plasmid, with or without pREP4X-p35 as indicated. Cell lysates were prepared and tested for their ability to inhibit CPP32-mediated cleavage of DEVD-AMC. Specified amounts of each extract were mixed and their ability to inhibit rCPP32 activity was assayed. Results are expressed as percentage of control which corresponds to the activity obtained when rCPP32 is combined with extract from control vector-transformed cells (pREP). The degree of inhibition obtained by addition of 2.5 ng of affinity-purified 6His recombinant p35 (rp35) is included for comparison. In D, 293 cells were transfected as in B except 1 μ g of pRcCMV-LacZ *LacZ* expression vector was included with each transfection. Twenty-four hours after transfection, cells were fixed and the presence of functional *LacZ* was detected with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Percentage of dead cells was determined as the ratio of round (dead) blue cells to flat (live) blue cells.

malian cells when undergoing apoptosis (Oberhammer *et al.*, 1993; Gromova *et al.*, 1995; Lagarkova *et al.*, 1995). Such DNA fragmentation however was not detected in *Dictyostelium*, suggesting that programmed cell death can occur in at least some types of protist cells in the absence of nonrandom DNA degradation.

Of relevance to this issue, genetic analyses of the nematode *C. elegans*, as well as investigations of some mutant mammalian cell lines, have suggested that genomic digestion is not a necessary requirement for programmed and apoptotic cell death in animal cells (reviewed in Ellis, 1991; Ucker, 1991).

The morphological features of yeast dying as a result of Bax or Bak expression were not unique to these proapoptotic Bcl-2 family proteins. For example, overexpression of the protein tyrosine phosphatase gene *pyp1⁺* in fission yeast resulted in a similar phenotype, but with a longer delay compared with Bax and Bak. Furthermore, cell death induced in the budding yeast *S. cerevisiae* by overexpression of histone H1 produces a very similar morphology (Miloshev *et al.*, 1994). Thus, the cell death processes induced by a variety of stressors, including Bax and Bak, may ultimately culminate in a similar morphological phenotype in yeast.

The cell death induced by Bax and Bak in *S. pombe* differed from Bax-induced cell death in mammalian cells in that no ICE/CED-3-like protease activities were detected and the baculovirus p35 protein could not protect fission yeast from the lethal effects of Bak and Bax. In the nematode, *C. elegans*, loss-of-function mutations in *ced-3*, which encodes a cysteine protease with homology to ICE, results in a failure of all 131 programmed cell deaths that normally occur in this animal during development (Yuan *et al.*, 1993). Conversely, loss-of-function mutations in the *ced-9* gene, the worm homologue of *bcl-2*, cause massive developmental cell death, which can be blocked by the baculovirus p35 protein (Hengartner *et al.*, 1992; Sugimoto *et al.*, 1994). Ectopic expression of the p35 protein in the eyes of transgenic flies also protects from cell death induced by overexpression of the cell death genes *reaper* and *hid* (Pronk *et al.*, 1996; White *et al.*, 1996). In human cell lines, apoptosis induced via the cytokine receptors Fas/APO-1 (CD95) and tumor necrosis factor receptor type I can also be prevented by p35 (Beidler *et al.*, 1995). The p35 protein plays an important role in the normal life cycle of baculoviruses, which depend at least in part on p35 for preventing cell death and thus allowing viral replication to occur in infected insect cells (Clem and Miller, 1994). The fact that p35 can rescue mammalian 293 cells from apoptosis induced by Bax expression but cannot suppress Bax- and Bak-induced cell death in *S. pombe* suggests that the downstream events leading to cell death in fission yeast when expressing Bax or Bak are distinct from those occurring after Bax overexpression in animal cells.

These observations raise the possibility that the ICE/CED-3 family cysteine proteases might have arisen at a relatively late point in the evolution of cell death suicide mechanisms, after the division of the plant and animal kingdoms. It should be noted, however, that other types of cell death-associated aspartases may be present in yeast which are both undetectable by the particular protease assays used here and are uninhibitable by p35. For example, cytolytic T-cells in humans and rodents produce a serine protease, granzyme B, with specificity for aspartic acid in the P1 position of substrates which is a potent inducer of

apoptosis but which is not inhibited by p35 (Bump *et al.*, 1995; Quan *et al.*, 1995; Tewari *et al.*, 1995). Also, it remains unknown at present whether all programmed or apoptotic cell deaths in animal species require the actions of ICE/CED-3-like aspartases.

The findings that 1) Bax can induce cell death in both fission and budding yeast and that 2) Bcl-X_L and Bcl-2 can rescue yeast from the lethal actions of this proapoptotic protein raise the possibility that at least some portions of the cell death mechanism controlled by Bcl-2 family proteins may be evolutionarily conserved even beyond animal species to simple unicellular eukaryotes and perhaps plants. The question then is to what extent the biochemical processes regulated by Bcl-2 family proteins in yeast resemble those that are relevant to cell death control in animal cells. Similar to reports on mammalian cells, we observed that a mutant version of Bcl-2 (G145A) which fails to heterodimerize with Bax was incapable of suppressing Bax- or Bak-induced cell death in the fission yeast *S. pombe*. Also, a variety of loss-of-function mutants of Bcl-2 that either do or do not retain Bax-binding activity were previously shown to be inactive at suppressing Bax-mediated lethality in the budding yeast *S. cerevisiae* (Hanada *et al.*, 1995). Moreover, as shown here, the Bak protein also functioned as a cell death inducer in *S. pombe*, despite the fact that it shares only 19% amino acid sequence homology with Bax (Kiefer *et al.*, 1995). In this regard, the recently reported three-dimensional structure of the Bcl-X_L protein has suggested that Bcl-2 family proteins may be pore-forming molecules with similarity to certain bacterial toxins such as diphtheria toxin and bacterial colicins (Muchmore *et al.*, 1996). Thus, the cell death mechanism induced in *S. pombe* and *S. cerevisiae* by Bax and Bak potentially may be a reflection of an intrinsic activity of these proteins that requires no further cooperating proteins from yeast.

Although these observations could be taken as evidence that Bax/Bak-induced cell death in fission and budding yeast has close parallels with mammalian systems, it is also possible that Bax and Bak trigger cell death through mechanisms that are biochemically dissimilar in yeast and animal cells. In this regard, we have been unable to identify homologues of Bcl-2 and Bax in yeast by low-stringency hybridization and two-hybrid screening of genomic libraries, raising the possibility that yeasts do not normally rely on such proteins for regulating cell survival and death. These caveats notwithstanding, the data reported here and elsewhere (Sato *et al.*, 1994; Hanada *et al.*, 1995; Greenhalf *et al.*, 1996) showing biological effects of Bcl-2 family proteins in yeast, when taken together with the recent x-ray crystallographic and NMR structural data for Bcl-X_L, suggest that yeast may provide a convenient background in which to perform certain types of structure-function analyses of Bcl-2 family proteins.

Moreover, these simple organisms may provide important opportunities for probing the evolutionary origins of programmed cell death mechanisms.

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Minireview

Death-Defying Yeast Identify Novel Apoptosis Genes

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Intensive study of processes that initiate, control, and execute the cell death program termed apoptosis has resulted in a wealth of new information regarding how cells contribute to their own demise. The picture emerging from these studies suggests a complex interplay between factors that promote cell death and those that prevent cell death, the end result being life or death of the cell (for reviews, see Horvitz et al., 1994 [1]; Salvesen and Dixit, 1997 [2] references therein). The most downstream components of the cell-death machinery identified so far are proteases known as caspases, a class of cysteine proteases that cleave substrates following aspartate residues. Most apoptotic processes, from those in the nematode *Caenorhabditis elegans* to those in humans, result in activation of these proteases leading to cell death (Figure 1). The activity of caspases can be regulated by a variety of cellular factors. Some, such as the *C. elegans* protein CED-4, the related mammalian protein Apaf-1, or the mammalian protein Fas, can activate caspases and are thus death promoters (Shaham and Horvitz, 1996 [3]; Salvesen and Dixit, 1997 [2]; Zou et al., 1997 [4]). Others, such as *C. elegans* CED-9 or mammalian Bcl-2 can inhibit activation of caspases. Yet other proteins, such as mammalian Bax, which is similar to Bcl-2, seem to promote cell death in part by inhibiting the activities of death-preventing proteins of the Bcl-2 family. Regulators such as Bax can, in turn, be inhibited by Bcl-2 (Korsmeyer et al., 1993 [5]) (Figure 1).

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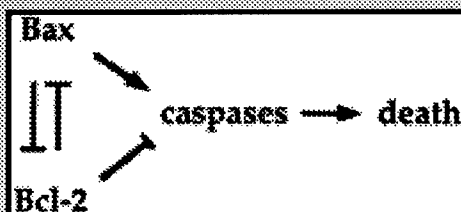


Figure 1. Regulation of Caspases and Cell Death by Bax and Bcl-2

Blunt arrows indicate inhibition. Pointed arrows indicate activation. Some reports suggest that Bax and Bcl-2 can also promote and prevent cell death, respectively, in a caspase-independent manner (see, for example, Xiang et al., 1996 [6]).

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Although some of the interactions among known cell-death components have been described, there are still major gaps in our understanding of the apoptotic death process. For example, the cellular targets of caspases that lead to cell death have not yet been fully described. In addition, the mechanisms by which death-preventing members of the Bcl-2 family inhibit caspase activation and how Bax and other

death-promoting relatives of Bcl-2 promote cell death also remain obscure.

Two papers published in the current issue of *Molecular Cell* (Xu and Reed, 1998 [2]; Matsuyama et al., 1998 [3]) provide a novel entry to understanding how Bax and Bcl-2 might function to promote and prevent apoptosis, respectively. The authors describe the identification of two new components of the cell-death pathway using the budding yeast *Saccharomyces cerevisiae*. Although budding yeast does not exhibit apoptosis or contain homologs of Bcl-2 or caspases, Bax and a closely related death-promoting protein, Bak, have been previously described to induce cell death both in this organism and in fission yeast. These deaths can be inhibited by coexpression of Bcl-2. Furthermore, expression of mutant versions of Bax or Bak that lack activity in mammalian cells do not result in yeast lethality (Zha et al., 1996 [4]; Ink et al., 1997 [5]). These observations suggest that the same properties that cause Bax to kill mammalian cells are required for it to kill yeast cells. Using a Bax gene whose expression is induced in the presence of galactose (pGal-Bax), Xu and Reed, 1998 [2] and Matsuyama et al., 1998 [3] searched for human cDNAs and yeast mutations, respectively, that prevent Bax-induced cell death in galactose-containing medium. Their results support the idea that yeast can be used as a vehicle for identifying metazoan cell-death factors.

Bax Inhibitor-1 (BI-1) Inhibits Mammalian Apoptosis

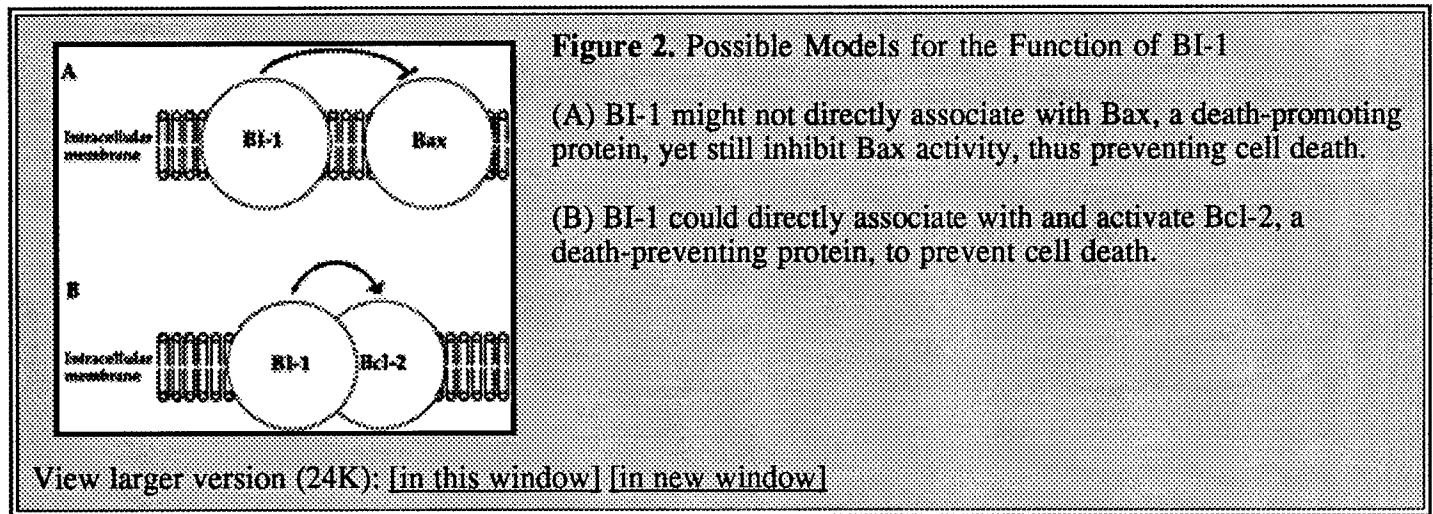
Xu and Reed, 1998 [2] transformed pGal-Bax-containing yeast cells with a human cDNA library in which cDNAs were fused to a constitutively active yeast promoter and isolated four cDNAs that prevent lethality on galactose. Three encoded the same protein, termed BI-1 (for Bax Inhibitor-1) and were studied further. The *BI-1* gene is identical to a previously isolated human gene of unknown function called *TEGT* (Testis Enhanced Gene Transcript). Homologs of BI-1 are found in both rat and mouse as well as in the plant *Arabidopsis thaliana*. Weak similarity was also found with a *C. elegans* gene, indicating that BI-1 is conserved in evolution.

To determine if BI-1 plays a role in mammalian apoptosis, the authors tested the ability of BI-1 to block cell death induced by Bax overexpression, growth factor deprivation, etoposide, and staurosporine treatment of cultured mammalian cells. They found that BI-1 could inhibit cell death to a similar extent as Bcl-2, suggesting that BI-1 might normally function to prevent mammalian apoptosis. Expression of antisense BI-1 RNA induced apoptosis in approximately 20% of cultured 293 cells, supporting the notion that BI-1 is a mammalian cell-death inhibitor. Evidence that BI-1 is a key regulator of mammalian apoptosis could be supplied by analysis of BI-1^{-/-} mice. Increased apoptosis in such knockout mice would provide strong support for the importance of BI-1 as an inhibitor of apoptosis.

The observation that BI-1 inhibits Bax-induced death in both mammalian cells and *S. cerevisiae* is remarkable. These results are a clear validation of the approach used to identify genes affecting Bax activity. The screen used by the authors does not appear to have been exhaustive, since one cDNA class isolated was represented by only a single cDNA. Furthermore, at least one gene that should have been isolated, namely *Bcl-2* (Figure 1), was not recovered. Thus, continued screening would likely reveal additional cell-death inhibitors.

The *BI-1* gene encodes a protein containing several putative transmembrane domains. Examination of BI-1 localization using GFP- or FLAG-tagged BI-1 revealed that these proteins are located primarily in the endoplasmic reticulum (ER) membrane and nuclear envelope. Weak staining was also detected in mitochondrial membranes. Interestingly, Bcl-2 had been previously localized to intracellular membranes (reviewed by Kroemer, 1997 [6]). Cell fractionation experiments performed by the authors reveal that tagged BI-1 and Bcl-2 colocalize, suggesting that these proteins might interact. Support for this notion was provided by both in vivo cross-linking and coimmunoprecipitation experiments.

How might BI-1 prevent cell death in mammalian cells? Based on the general scheme shown in Figure 1, the primary action of BI-1 might be either to inhibit Bax or to stimulate Bcl-2 (Figure 2). If BI-1 inhibits Bax activity, it is unlikely to do so by direct association since the two proteins do not coimmunoprecipitate (Figure 2A). BI-1 could inhibit Bax activity without direct association, for example, by activating an inhibitor of Bax. The ability of BI-1 to associate with Bcl-2 in vivo suggests that Bcl-2 could be such an inhibitor. Alternatively, BI-1 could activate Bcl-2 to inhibit death in a Bax-independent manner (Figure 2B). Whether BI-1 activates Bcl-2 could be tested by determining whether overexpression of BI-1 prevents the death of cells lacking Bcl-2.



Although overexpression of BI-1 prevented cell death induced by growth factor depletion and other conditions (see above), it was unable to prevent apoptosis resulting from activation of Fas, a cell-surface receptor that promotes cell death when activated. The Fas receptor is thought to promote cell death by activating a cascade of caspases and can function in a Bcl-2-independent manner (Vaux and Strasser, 1996 [2], Salvesen and Dixit, 1997 [3]). BI-1 is thus not a universal cell death inhibitor and apparently affects some modes of apoptosis but not others.

Mitochondrial F_0F_1 -ATPase— a Possible Role in Bax-Induced Apoptosis

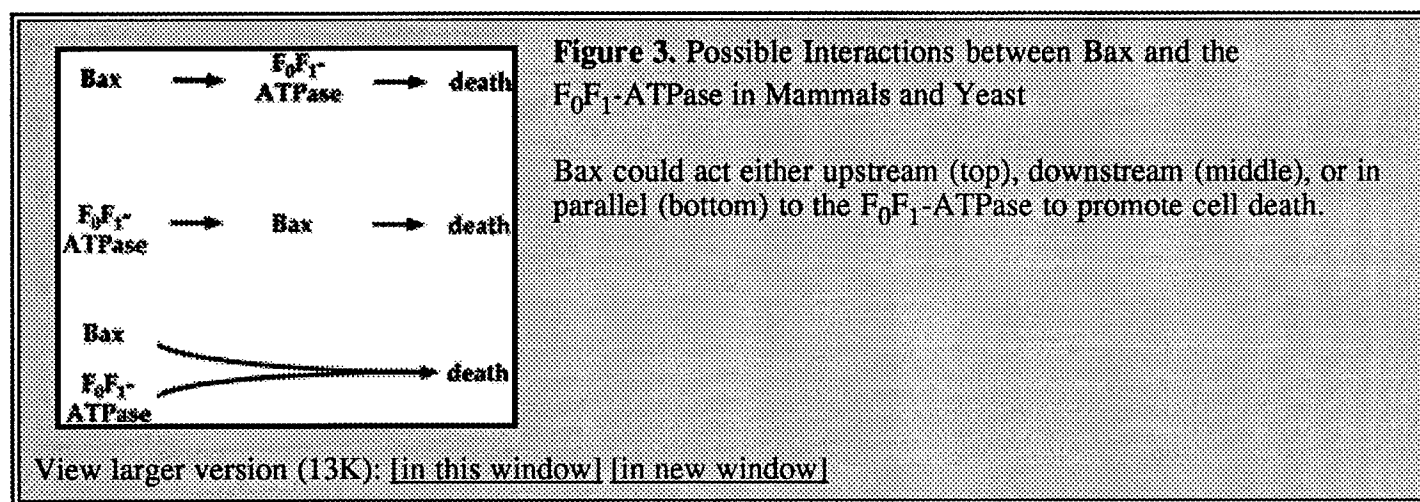
In order to identify components required for Bax-induced cell death in yeast, Matsuyama et al., 1998 [4] screened for yeast mutants that do not exhibit pGal-Bax-induced death on galactose-containing medium. The authors isolated one recessive Bax-resistant yeast mutant and cloned the relevant gene by standard methods of genetic complementation. The gene (*ATP4*) encodes a subunit of the yeast F_0F_1 -ATPase, a proton pump required for aerobic respiration that is located in the inner mitochondrial membrane. Interestingly, recent evidence has suggested an important role for mitochondria in some forms of apoptosis. Mitochondrial cytochrome c can be detected in the cytoplasm of mammalian cells undergoing apoptosis, and, in association with Apaf-1, a protein related to *C. elegans* CED-4, can activate certain caspases in vitro (Zou et al., 1997 [5]). In addition, several members of the Bcl-2 family including Bcl-2 and Bax have been shown to associate with the outer mitochondrial membrane in mammalian cells. Overproduction of Bcl-2 can prevent both cytochrome c release and apoptosis in mammalian cells (reviewed by Reed, 1997 [6]). When overexpressed in yeast, Bax is primarily associated with the outer mitochondrial membrane and can also cause cytochrome c release (Manon et al., 1997 [7]), suggesting that Bax-induced death in yeast might share mechanistic similarities with mammalian apoptosis.

A variety of observations support the argument that the F_0F_1 -ATPase is required for Bax-induced killing in yeast. First, Matsuyama et al. demonstrated that interruption of the *ATP4* coding sequence also suppressed Bax-mediated killing, thus confirming the results of their screen. Second, a mutation in a different subunit of the F_0F_1 -ATPase also inhibited Bax-induced killing. Third, yeast cells exposed to oligomycin, a potent inhibitor of the F_0F_1 -ATPase, did not undergo Bax-induced death. Fourth, mutant yeast cells unable to respire via mitochondrial oxidative phosphorylation yet containing an intact F_0F_1 -ATPase were not protected against Bax killing. These experiments strongly suggest that the activity of the F_0F_1 -ATPase, and probably not respiration in general, is required for killing of yeast cells by Bax.

As with the paper by Xu and Reed, 1998 [8], Matsuyama et al. tested the relevance of their discoveries in mammalian cells, where Bax normally acts. Strikingly, the authors showed that death of mammalian cells induced by Bax could be partially inhibited by oligomycin but not by antimycin, a drug affecting mitochondrial respiration. Caspase activation during Bax-induced cell death was also inhibited by oligomycin.

These results mirror the yeast experiments and suggest that Bax activity in mammalian cells may at least partially require a functional F_0F_1 -ATPase. It would be interesting to test if the F_0F_1 -ATPase is required for cell death other than that induced by Bax in both mammals and yeast. If so, this protein complex might define a key step of the cell death process.

The F_0F_1 -ATPase might promote Bax-induced killing in mammals and in yeast by activating Bax, by being a target of Bax activity, or by acting in parallel to Bax (Figure 3). The data presented by the authors do not allow discrimination among these possibilities. Bax and other Bcl-2 family members have been shown to form pores when inserted into membranes (Schlesinger et al., 1997 [references therein]). It is thus possible that the interaction between Bax and the F_0F_1 -ATPase, a proton pump, reflects a role for ion transporters in apoptosis. The F_0F_1 -ATPase is localized to the inner mitochondrial membrane. Intriguingly, cytochrome c is located in the intermembrane space of mitochondria and can interact with some proteins of the mitochondrial inner membrane. Thus, if cytochrome c release into the cytoplasm plays a causal role in mammalian and yeast cell death, then the F_0F_1 -ATPase might be necessary for this release.



Yeast and Apoptosis

The requirement for the F_0F_1 -ATPase in Bax-induced death of yeast cells suggests that yeast may contain additional conserved components of an apoptotic pathway. Reports in the literature suggest that death of either budding or fission yeast in response to overexpression of Bak or mutation of the budding yeast *CDC48* gene result in chromatin condensation, DNA degradation and membrane blebbing—cellular features that are hallmarks of metazoan apoptosis (Ink et al., 1997 []; Madeo et al., 1997 []). In addition, Bcl-2 has been reported to improve viability of yeast mutants defective in *SOD1* under some growth conditions. *SOD1* codes for the cytoplasmic Cu-Zn superoxide dismutase, which functions in detoxifying free radicals. These results are similar to those in mammalian cells and suggest that Bcl-2 might have antioxidant properties (Long et al., 1997). It is thus possible that molecular pathways leading to certain subphenotypes of apoptotic cells are present in yeast cells. Conservation of a number of functional modules between yeast and mammals is certainly striking. Yeast cells harbor modules such as MAP kinase cascades, GTPases and their exchange factors, and G proteins and their regulators, which have shed light on analogous mammalian processes even though the components may be used in different functional contexts. Similar modules might exist in metazoan apoptotic pathways.

Even though yeast does not contain obvious homologs of known metazoan cell-death regulators, there are a variety of strategies that could be used to look for yeast products involved in yeast cell death. Proteins that inhibit cell death (BI-1 analogs) could be identified by looking for yeast genes whose overexpression inhibits Bax-mediated killing. Such proteins could also be discovered by looking for genes whose inactivation results in lethality that is suppressed by expression of a metazoan death inhibitor such as Bcl-2. Conserved components of a cell death pathway (Bax analogs) could also be identified by screening for yeast genes whose

expression kills yeast cells in an F_0F_1 -ATPase-dependent manner. Finally, experiments similar to those described in Xu and Reed, 1998 [] and Matsuyama et al., 1998 [] could be carried out on yeast cells induced to die by expression of metazoan death promoters other than Bax.

The current picture of apoptosis has been developed from studies of a variety of organisms—nematodes, flies, and animal cells. The papers reviewed here suggest that using yeast to study apoptosis might be richly rewarding. Studying metazoan cell death in yeast may thus turn into a major growth (death) industry.

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