The Mitochondrial F$_{0}$F$_{1}$-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$_{0}$F$_{1}$-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$_{0}$F$_{1}$-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 primarily with 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 necrotic 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 vacuolarization and apparent disruption of mitochondria (Link 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 Bri-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 (Alimena 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
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), Trypanosome brucei rhodesiense (Welburn et al., 1996), Trypanosome 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) × 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 μg) 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,F'-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...
Bax Requires a Mitochondrial Proton Pump

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\(^1/2\) 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 with Myy290 (ATP4\(^2\)) were transformed with pGilda (C) or pGilda-Bax. The cells were grown in glucose-based medium and then cultured 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 ± 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\(^1\)/ATP4\(^2\) 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.

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 4. ATPδ-Deficient but Not rho− petite Yeast Are Bax-Resistant

Strain CGY1 yeast that harbor a URA3-disrupted ATPδ 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 ± SE; n = 3; some SE symbols are obscured by symbols). In (C), lanes 1/2 and lanes 3/4 represent rho− and ATPδ-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, loss of the ϵ subunit of the F0F1-ATPase subunit had been inactivated by URA3 insertional mutagenesis (Giraud and Velours, 1994). Unlike the ATP4 mutant, both rho− and ATP-ϵ-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 F0F1-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-ϵ protein results in deficient function of both the F0 and F1 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

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 F0F1-ATPase. Thus, to explore whether the proton pump is also required for optimal function of Bax in mammalian cells, one is limited...
Bax Requires a Mitochondrial Proton Pump

Figure 5. The F$_{0}$F$_{1}$-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 µg of pcDNA-Bax (Bax) or control pcDNA plasmids with 1 µg of pEGFP, the culture medium was exchanged with fresh medium containing or lacking 0, 0.1, 1, or 10 µM oligomycin or 10 µM 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 ± 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 mClCCP for 15 min prior to incubation with DiOC6.

(C) Lysates derived from cells that had been cultured with or without 10 µM of oligomycin were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1996). 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 antibody to tubulin. Typical substrate hydrolysis progress curves are shown (representative of three experiments). The blot was subsequently reprobed with anti-tubulin antibody to verify loading of equivalent amounts of total protein.

90% trypan blue dye exclusion in control cultures was dependent on the mitochondrial pH gradient, 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 µM oligomycin preventing approximately half of the Bax-induced cell death. DiOC$_{6}$-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 mClCCP confirmed that the DiOC$_{6}$ 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 $\Delta$Ψ (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 µM 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 µM oligomycin (32 ± 3 nmol/mg protein versus 34 ± 3 nmol/mg protein) and to within ~75% of control levels for cells treated with 10 µM 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$_{50}$ ~ 5 µM) (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 µM, ouabain had no influence on Bax function (not shown). Based on the above results,
of 37±39 in these human cells. Their activities are modulated by pH (Antonsson et al., 1996; Sabbatini et al., 1997). We therefore employed pH. In addition, most Bcl-2 family proteins including Bax.

The induction of apoptosis in baby rat kidney (BRK) cells are reportedly higher at neutral than at acidic pH, oligomycin and to within $z$ to maintain ATP levels through glycolysis. Changes of pH at the surface of the inner membrane pump is either required for optimal function of Bax in In this regard, the channels formed in vitro by Bax, as

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using anti-Bax antiserum with ECL-based detection. The same blot the F0F1-ATPase contribute to Bax-induced cell death? was reprobed with anti-tubulin. Immunoblot analysis of BRK cells demonstrated mediated dimerization could play an important role in

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that neither oligomycin nor antimycin A (10 $\mu$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 F0F1-ATPase proton pump.

Discussion

Here we present genetic evidence that the mitochondrial F0F1-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 F0F1-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 F0F1-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 F0F1-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 (Krajewska 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 $\alpha$ helices of Bax through the lipid bilayer (reviewed in Reed, 1997).

Barring a direct physical interaction, how then might the F0F1-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 $\lambda$, 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 $\alpha$5 and $\alpha$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 F0F1-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 $\alpha$5 and $\alpha$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 $\alpha$ 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

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**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 at permissive temperature of 32°C where p53 is inactive. As in the prior experiments, these cells were grown in high glucose medium that neither oligomycin nor antimycin A (10 $\mu$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 F0F1-ATPase proton pump.

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**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 $\mu$M oligomycin or 10 $\mu$M antimycin A. In (A), the percentage of dead cells was determined by DAPI staining 12 hr after shifting to 32°C (mean ± 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 $\mu$M oligomycin or 10 $\mu$M antimycin for 12 hr, normalized for total protein content (5 $\mu$g/lane), and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobed with anti-tubulin.

Therefore, we conclude that the F0F1-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.
modulating interactions of Bcl-2 and Bcl-X, 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$_0$F$_1$-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$_0$F$_1$-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$_0$F$_1$-ATPase proton pump would be predicted to run in reverse, thus consuming ATP and alkalinizing the matrix by extruding protons. Since alkalinization 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; Ichas et al., 1997; Jung et al., 1997), the F$_0$F$_1$-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$_0$F$_1$-ATPase, since Fas can still induce apoptosis in cells exposed to oligomycin in high-glucose media. Though this observation clearly suggests that the F$_0$F$_1$-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$_0$F$_1$-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 Xhol sites of pgilda (gift of C. Kaiser [MIT]). The YCp50 plasmid (ATCC 37419) and yeast genomic library (ATCC 37415) 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., 1995). 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 colonies were picked from plates and grown in MM-A with galactose prior to transformation with the ADH1 promoter-containing plasmid pEG202-Bax (Sato et al., 1994) and selection on MM-A/2% glucose plates. Mutant yeast were mated with Mzy290 strain (MAT a, 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 reporter.

**Complementation Cloning**

BRM1 cells was transformed with the GAL10 promoter-containing plasmid pgilda-Bax and grown in MM-A with glucose to a density of $2 \times 10^4$ cells/ml. These cells were then transformed using a LiOAc method with 2 μg of a yeast genomic library in YCp50 (ATCC) and 20 μg of salmon sperm DNA transformation efficiency $1 \times 10^6$ to $2 \times 10^6$ per μg DNA). Transformed cells were first plated on MM-A with glucose and then replica-plated to MM-A with galactose. From $\sim 2 \times 10^8$ 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' CAGCATGCGGCGCATGCGCCGCG-3' and 5' GAGTGATGCCGGCCACGATGCGTCCG-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 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 μM oligomycin and half with ethanol 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 10^5 cells in 3.6 ml of medium per 6 cm diameter dish or 3 x 10^5 cells in 10 ml per 10 cm dish. Fresh medium was exchanged and 4 hr later the cells were transfected with 10 μg of pcDNA3-Bax versus parental pcDNA3 plasmid (10 cm dishes) or were cotransfected with 1 μg of pEGFP (Clontech Laboratories, Inc.) and either 9 μg of pcDNA3-Bax or pcDNA3 control plasmid (6 cm dishes). Four hours after the transfection, the medium was changed with fresh media containing 10 μM oligomycin, 10 μM antimycin A, or 0.1% ethanol (solvent). After culturing for an additional 3 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., 1995).

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 x 10^4 cells/ml in 96-well flat bottom plates or at 10^5 cells/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 lamp extract (Luciferase-luciferin, Sigma) with a luminometer. Data were normalized relative to total protein content of cell lysates.

**Measurements of Mitochondrial Δψ**

Mitochondrial Δψ was measured using DiOC6 (Molecular Probes, Inc.) as described (Castedo et al., 1996).

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