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Structure-Function Comparisons of the Proapoptotic Protein Bax in Yeast and Mammalian Cells

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Expression of the proapoptotic protein Bax under the control of a *GAL10* promoter in *Saccharomyces cerevisiae* resulted in galactose-inducible cell death. Immunofluorescence studies suggested that Bax is principally associated with mitochondria in yeast cells. Removal of the carboxyl-terminal transmembrane (TM) domain from Bax [creating Bax (ΔTM)] prevented targeting to mitochondrial and completely abolished cytotoxic function in yeast cells, suggesting that membrane targeting is crucial for Bax-mediated lethality. Fusing a TM domain from Mas70p, a yeast mitochondrial outer membrane protein, to Bax (ΔTM) restored targeting to mitochondria and cytotoxic function in yeast cells. Deletion of four well-conserved amino acids (IGDE) from the BH3 domain of Bax ablated its ability to homodimerize and completely abrogated lethality in yeast cells. In contrast, several Bax mutants which retained ability to homodimerize (ΔBH1, ΔBH2, and Δ1-58) also retained at least partial lethal function in yeast cells. In coimmunoprecipitation experiments, expression of the wild-type Bax protein in Rat-1 fibroblasts and 293 epithelial cells induced apoptosis, whereas the Bax (ΔIGDE) mutant failed to induce apoptosis and did not associate with endogenous wild-type Bax protein. In contrast to yeast cells, Bax (ΔTM) protein retained cytotoxic function in Rat-1 and 293 cells, was targeted largely to mitochondria, and dimerized with endogenous Bax in mammalian cells. Thus, the dimerization-mediating BH3 domain and targeting to mitochondrial membranes appear to be essential for the cytotoxic function of Bax in both yeast and mammalian cells.

Cell death plays an important role in many physiological processes, including removal of redundant cells during development, elimination of autoreactive lymphocytes from the immune system, and tissue homeostasis in essentially all organs in which cell division occurs (reviewed in references 12, 16, 58, 76, and 80). Dysregulation of normal cell death mechanisms also figures prominently in several diseases (reviewed in references 7, 32, 58, 59, 73, and 80). In many but not all cases, the cell deaths that occur under normal physiological circumstances involve characteristic morphological changes that are broadly known as apoptosis. These morphological changes, which include nuclear fragmentation, chromatin condensation, cell shrinkage, plasma membrane blebbing, and budding off of cellular fragments, are distinct from the cellular and organellar swelling and rupture that are typically seen in the setting of necrosis (82, 83).

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 an evolutionarily conserved pathway for physiological cell death and apoptosis, with some members functioning as suppressors of apoptosis and others functioning as promoters of cell death (reviewed in references 53, 56, 58, and 75). Interestingly, though best known for its ability to regulate apoptotic cell death, the antiapoptotic protein Bcl-2 has also been shown to be capable of suppressing necrotic cell death under some circumstances (28, 29, 66, 69), 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 biochemical mechanisms by which Bcl-2 family proteins control cell life and death remain enigmatic, principally because their predicted amino acid sequences have no significant homology with other proteins. The nuclear magnetic resonance and X-ray crystal structures of the Bcl-2 family protein Bcl-X_L, however, suggest structural similarity to bacterial toxin, pore-forming proteins such as the B subunit of diphtheria toxin, and colicin A (50). The functions of Bcl-2 family proteins appear to be dependent at least in part on their ability to interact with each other through a complex network of homo- and heterodimers (4, 19, 55, 61, 65, 86).

In this report, we have focused on a structure-function analysis of the Bax-α protein, a 21-kDa proapoptotic member of the Bcl-2 protein family (55). Bax-α is widely expressed in mammalian tissues (34). Loss of Bax function through gene disruption in mice leads to hyperplasias in several tissues in vivo, suggesting a need for Bax in some of the cell turnover mechanisms required for normal tissue homeostasis (31). Expression of Bax is selectively induced in vivo by γ irradiation in radiosensitive tissues that experience apoptotic cell death after genotoxic injury (30). Moreover, the *BAX* gene is a direct transcriptional target of p53 (47), suggesting an important role of Bax in radio- and chemoresponses of tumors (89). Consistent with this idea, reductions in Bax protein levels which occur in about one-third of metastatic breast cancers have been correlated with poor patient responses to combination chemotherapy and shorter overall survival (33). Striking elevations in Bax protein levels are also induced in several types of central nervous system neurons after transient cerebral ischemia and in embryonic neurons when deprived of nerve growth factor, implying a potentially important role for this protein in some

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of the neuronal cell deaths that occur during stroke or as a result of neurotrophic factor deprivation (35, 60).

The mechanisms by which p21-Bax promotes apoptosis are presently unclear. Like many other members of the Bcl-2 protein family, the Bax protein has a stretch of hydrophobic amino acids located at its carboxyl terminus which presumably allows the protein to posttranslationally insert into membranes. Though the precise location of the p21-Bax protein in cells has not been determined, immunohistostaining and immunofluorescence studies indicate a mostly cytosolic location, in association perhaps with organelles (14, 18, 24, 34, 36, 48, 84). All Bcl-2 family proteins examined to date have been shown to reside at least in part in mitochondrial membranes (14, 24, 36, 38, 48, 84). In the case of Bcl-2, the association with mitochondria occurs via the outer rather than inner membrane, with the protein oriented toward the cytosol (36, 38, 48).

In addition to a C-terminal transmembrane (TM) domain, p21-Bax also contains three domains, BH1, BH2, and BH3, which are well conserved among Bcl-2 family proteins. Recent studies indicate that Bax homodimerizes with itself and heterodimerizes with Bcl-2 and other antiapoptotic Bcl-2 family proteins in a BH3-dependent manner (18, 88). The functional significance of these homo- and heterodimerization phenomena has not been determined for Bax. With Bcl-2, however, mutations in this antiapoptotic protein which impair its ability to dimerize with Bax have been shown to abrogate its function, implying that Bcl-2–Bax heterodimerization is important for suppression of cell death (86).

Recently, it has been shown that when expressed in the budding yeast *Saccharomyces cerevisiae*, the Bax protein confers a lethal phenotype (17, 61, 62). Analogous to findings of studies performed with mammalian cells, antiapoptotic members of the Bcl-2 protein family, including Bcl-2, Bcl-X_L, and Mcl-1, are able to suppress the lethal function of Bax in yeast cells, whereas the proapoptotic protein Bcl-X_S and various deletion mutants of Bcl-2 which are nonfunctional in mammalian cells do not (4, 17, 19, 61). Interestingly, even some mutants of Bcl-2 which retain the ability to bind Bax but which are deficient in antiapoptotic activity in mammalian cells are also unable to suppress Bax-mediated lethality in yeast cells (19). Taken together, these observations suggest that at least some aspects of Bax and Bcl-2 protein function may be conserved in yeast cells, thus prompting us to compare some of the structure-function activities of the Bax protein in the unicellular eukaryote *S. cerevisiae* with those in mammalian cells.

MATERIALS AND METHODS

Plasmids. A ~0.6-kbp murine *bax* cDNA subcloned into the *Eco*RI site of pSKII (Stratagene, Inc.) (46) was liberated by *Eco*RI digestion, the ends were blunted with Klenow fragment, and the fragment was subcloned by blunt-end ligation into YEp51 at a blunted *Sal*I site downstream of the *GAL10* promoter. Clones with the *bax* cDNA inserted in sense orientation were identified by restriction digestion and confirmed by DNA sequencing.

The wild-type *bax* cDNA and various *bax* mutants were subcloned between the *Eco*RI and *Xho*I sites in the two-hybrid plasmid pEG202 in frame with the NH_2-terminal LexA DNA binding domain sequences (13). The wild-type Bax (encodes all 192 amino acids), Bax (ΔN) (mutant missing the first 58 amino acids), Bax (ΔTM) (missing residues 172 to 192), and TM (contains only Bax residues 172 to 192) were created by one-step PCRs using pSKII-*bax* as the template and 5′-GGGAATTCGCGGTGATGGACGGGT-3′ [Bax and Bax (ΔTM)], 5′-GGGAATTCGCGGTGATGCTGAGCGAGTGTCTCCGGCGAA TT-3′ [Bax (ΔN)], or 5′-GGAATTCGCGGTGATGACAGTGACCATCTTTG TGGCTGG-3′ (TM) as the forward primer and either 5′-CTCTCGAGTCAG CCCATCTTCTTCCAGATGGT-3′ [Bax, Bax (ΔN), and TM] or 5′-CTCTCG AGTCACTGCCATGTGGGGGTCCCGAA-3′ [Bax (ΔTM)] as the reverse primer. The Bax mutant plasmids Bax (ΔIGDE), Bax (ΔBH1), and Bax (ΔBH2) were prepared by the two-step PCR method of Ho et al. (22), using pSKII-*bax* as the template and the following mutagenic primers in combination with the wild-type Bax forward and reverse primers described above: Bax (ΔIGDE), 5′-GAGTGTCTCCGGCGACTGGATAGCAATATGGAGCTG-3′ (forward) and 5′-CATATTGCTATCCAGTCGCCGGAGACACTCGCTCAG-3′ (reverse); Bax (ΔBH1), 5′-GCAGCTGACATGTTTGCTCTCTTCTACTTTGCTA GCAAACTGG-3′ (forward) and 5′-CTAGCAAAGTAGAAGAGAGCAAAC ATGTCAGCTGCCACC-3′ (reverse); and Bax (ΔBH2), 5′-GTGAGCGGCTG CTTGTCGGCCTCCTCTCCTACTTCGG-3′ (forward) and 5′-GAAGTAGG AGAGGAGGCCGACAAGCAGCCGCTCACGGAG-3′ (reverse). The Bax (ΔIGDE) (ΔTM) plasmid was prepared as described above except substituting the Bax (ΔTM) reverse primer for the wild-type Bax reverse primer. This cDNA was subcloned from pEG202 into pJG4-5 by digestion with *Eco*RI and *Xho*I.

To create the chimeric plasmid pEG202-Bax/Mas70, a cDNA encoding Bax residues 1 to 171 was generated by using pSKII-*bax* template DNA and the wild-type Bax forward primer described above with 5′-GGTTGCCAAAATGGCCT GCCATGTGGGGGTCCCGAA-3′ as the reverse primer. In addition, a cDNA encoding the Mas70p TM domain (residues 11 to 29) was generated by using plasmid YEplac181-Mas70 (20) as a template and 5′-GACCCCCACATGGCA GGCCATTTTGGCAACCGTTGCTG-3′ and 5′-CTCTCGAGTCACTTCTTG TAATAATAGTAGGCACCGATGG-3′ as the forward and reverse primers, respectively. These two gel-purified PCR products then combined in a final PCR using wild-type Bax forward primer and the Mas70p TM reverse primer. Plasmids pEG202-Fas, pEG202-lamin, pJG4-5-Ha-Ras, pJG4-5-Bcl-2 (ΔTM), and pJG4-5-lamin have been described previously (19, 61, 63).

Plasmid pHA-Shin contains three tandem copies of a hemagglutinin (HA) tag with an accompanying start codon and Kozak consensus sequence for translation in pcDNA-3 (Invitrogen, Inc.), subcloned downstream of the cytomegalovirus promoter between the *Hin*dIII and *Nco*I sites. The *bax* sequences in pEG202-Bax, pEG202-Bax (ΔTM), and pEG202-Bax (ΔIGDE) were excised by digestion with *Eco*RI and *Xho*I and subcloned in frame with the HA tag-encoding sequences in *Eco*RI-*Xho*I-digested pHA-Shin. The Bax (ΔTM) cDNA was also subcloned into the green fluorescent protein (GFP) plasmid pS65T (Clontech, Inc.) between the *Bgl*II and *Eco*RI sites. The proper construction of all plasmids was confirmed by DNA sequencing.

Yeast cell culture and transformations. All strains were maintained on minimal medium (MM) supplemented with essential amino acids (MM-A) and additional amino acids as required for maintenance of plasmid selectable markers. The following strains were used for analysis of Bax effects on cytotoxicity: EGY48 (*MAT*α *trp1 ura3 his3 leu2*::*plexAop*$_6$-*leu2*), EGY191 (*MAT*α *trp1 ura3 his3 leu2*::*plexAop*$_1$-*leu2*), MYY291 (*MAT*α *his3 ura3 leu2*), L40 (*MAT***a** *trp1 leu2 his3 ade2 LYS2*::*lexAop*$_4$-*HIS3 URA3*::*lexAop*$_8$-*lacZ*), and BF264-15Dau (*MAT*α *ade1 his2 leu2 trp1 ura3*). Transformations were performed by the lithium acetate method, using 1.5 μg of plasmid DNA and 5 μg of sheared, denatured salmon sperm (carrier) DNA. For transformations with YEp51 and pEG202-based plasmids, cells were plated on leucine-deficient and histidine-deficient MM-A, respectively.

For experiments with cells containing YEp51 or YEp51-Bax, a single colony of transformed cells growing on leucine-deficient MM-A supplemented with 2% (wt/vol) glucose was grown in 10 ml of medium with vigorous aeration at 30°C to an optical density at 600 nm (OD_{600}) of ~0.8 to 1.0. Cells were pelleted by centrifugation (1,000 × *g*) for 10 min and resuspended in 10 ml of leucine-deficient MM-A with either 2% glucose or 2% galactose–1% raffinose. After culturing for various times (0 to 4 days), an aliquot of cells was removed for trypan blue dye exclusion assay, counting ~400 total (live and dead) cells. Alternatively, the cell density of cultures was determined by counting unstained cells, and 10^3 cells were spread on either glucose- or galactose-containing leucine-deficient MM-A plates. Plates were cultured at 30°C for 4 days, and the number of colonies was counted.

Two-hybrid assays. Protein-protein interactions were evaluated by yeast two-hybrid assay essentially as described in detail previously, using either EGY48 cells for *LEU2* reporter gene assays or EGY191 cells for *lacZ* reporter gene assays, in conjunction with plasmids pEG202 (LexA DNA binding domain) and pJG4-5 (B42 transactivation [TA] domain) (13, 19, 61). Growth on leucine-deficient plates was scored 4 days after spotting cells on MM plates containing 2% galactose–1% raffinose to induce expression of the TA domain-containing proteins from the *GAL1* promoter in pJG4-5. Cells spotted on MM-glucose plates served as negative controls. Filter assays were similarly performed for β-galactosidase measurements, using cells plated on either galactose- or glucose-containing MM supplemented with 20 μg of leucine per ml. Colorimetric results were photographed after 0.5 to 2 h.

Immunoblot assays. Yeast cells were grown to an OD_{600} of ~1 to 2. Cells were then collected by centrifugation and washed with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in ~3 volumes of ice-cold lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.1 sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, protease inhibitors [57]) in Eppendorf microcentrifuge tubes. An equal volume of acid-washed, chilled glass beads (0.5-mm diameter) (Sigma, Inc.) was added, and the tubes were vortexed vigorously for 2 min. The beads were pelleted at 16,000 × *g* for 10 min at 4°C, and the supernatant was transferred to a fresh tube. Samples were normalized for protein content by the bicinchoninic acid method (Pierce, Inc.), and 15 to 30 μg was loaded per lane into SDS–12% polyacrylamide gels. After transfer to nitrocellulose membranes, blots were preblocked and antigen detection was performed as described previously, using 0.1% (vol/vol) anti-LexA or anti-Bax rabbit antiserum (34, 71) followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG). Antibody detection was accom-

plished by an enhanced chemiluminescence method (Amersham, Inc.) with exposure to X-ray film (XAR; Kodak, Inc.).

Immunofluorescence. Indirect immunofluorescence was performed as described previously (42, 43). Briefly, yeast cells were fixed by addition of formaldehyde to 4% (wt/vol) and shaken gently for 30 min at room temperature. Cells were collected by centrifugation, resuspended in 1/10 volume of buffer A (50 mM phosphate buffer, 35 mM KH_2PO_4, 15 mM K_2HPO_4, 0.5 mM $MgCl_2$ [pH 6.5]) containing 4% formaldehyde and shaken for an additional 30 min. Cells were then washed three times with buffer A containing 1.2 M sorbitol, and cell wall material was removed by incubation at room temperature in buffer A containing 1.2 M sorbitol, 27.5 mM β-mercaptoethanol, and 0.1 mg of Zymolyase 20T per ml. Cells were then washed twice with PBS containing 2 mg of bovine serum albumin (BSA) per ml (PBS-BSA) at room temperature and three times for 5 min each at −20°C with 0.1 ml of acetone. Ten to twenty microliters of the final acetone cell suspension was then spotted onto polylysine-coated slides. Slides were blocked by incubation in fresh PBS-BSA for 1 h with three buffer changes. PBS-BSA was replaced with 10 μl of primary antibody mix (0.073 μg of rat IgM monoclonal anti-Bax [Pharmingen, Inc.] per ml) and a 1:100 dilution of mouse IgG monoclonal anti-OM14 ascites fluid (42, 60) in PBS-BSA for 2 h. Each well was washed eight times with PBS-BSA, and 10 μl of secondary antibody solution (rhodamine isothiocyanate-goat anti-rat [IgM] and fluorescein isothiocyanate-donkey anti-mouse [IgG, heavy and light chains] from Jackson Laboratories, each at 30 μg/ml in PBS-BSA) was added for 1 h. After another eight washes, 10 μl of a 1-μg/ml solution of 4′,6-diamidino-2-phenylindole (DAPI) in PBS-BSA was added for 10 min. Wells were again washed eight times with PBS-BSA and allowed to air dry. All antibody and DAPI incubations were performed in a dark, humidified chamber. After mounting in a solution containing 1 mg of *p*-phenylenediamine per ml, 0.1× PBS, and 90% (vol/vol) glycerol, slides were viewed under fluorescence. Images were viewed under a 100× oil immersion objective, captured by a charge-coupled device camera after a further 5× magnification, and printed on Sony thermal imaging film.

Mammalian cell transfection and apoptosis assay. Rat-1 cells were maintained between 10 to 90% in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum, 1 mM L-glutamine, and antibiotics. Cells at 50% confluence in 24-well plates were transfected with 0.25 μg of pCMV-β-gal and 0.6 μg of plasmid pHA-Shin, pHA-Bax, pHA-Bax (ΔTM), or pHA-Bax (ΔIGDE) mixed with 5 μg of Lipofectamine (GIBCO/BRL, Inc.). After 24 h, cell monolayers were washed twice with PBS, and the adherent cells were fixed in 2% paraformaldehyde–0.1% glutaraldehyde in PBS. β-Galactosidase activity was detected by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate as described previously (37). The number of blue cells was counted by microscopic examination.

293 cells (~5×10^5) were cultured in 60-mm-diameter dishes in 3 ml of DMEM containing 10% horse serum (Donor Equine Serum, Tulare, Calif.) for 12 to 20 h. The cells were then transfected with 7 μg of pcDNA3-HA-Bax, pcDNA3-HA-Bax (ΔTM), pcDNA3-HA-Bax (ΔIGDE), or parental pcDNA3-HA plasmid DNA by a calcium phosphate precipitation method. Seven hours later, the transfection medium was replaced with fresh prewarmed (37°C) DMEM, and the cells were cultured for another 28 h. Floating cells in culture supernatant were recovered by centrifugation of the culture medium at 400 × *g* for 10 min. The adherent cells were recovered by trypsinization, pooled with floating cells, and washed once with the medium before fixation in 3.7% paraformaldehyde–PBS for 10 min. After one wash with PBS (pH 7.4), cells were resuspended in 1 ml of PBS containing 1 μg of DAPI, incubated for 10 min at room temperature, and washed three times with PBS. The percentage of cells with fragmented nuclei was determined by viewing with a fluorescence microscope.

Coimmunoprecipitation experiments. 293 cells (2×10^6) were cultured in 10 ml of DMEM containing 10% horse serum overnight. The cells were then transfected with 20 μg of each of pcDNA3-HA-Bax, pcDNA3-HA-Bax (ΔTM), pcDNA3-HA-Bax (ΔIGDE), or parental pcDNA3-HA vector by a calcium phosphate precipitation method. Approximately 60 h later, the cells were lysed in 0.3 ml of NP-40 lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% NP-40) containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml) and aprotinin (5 μg/ml). Immunoprecipitations were performed by incubation with 20 μl of protein G-Sepharose beads preabsorbed with 4 μg of anti-HA mouse monoclonal antibody (12CA5; Boehringer Mannheim) at 4°C for 3 h. After three washes in 1.5 ml of NP-40 lysis buffer, immune complexes were subjected to SDS-polyacrylamide gel (14%) electrophoresis (PAGE)–immunoblot analysis using anti-HA or anti-human Bax antiserum (33). Antibody detection was accomplished by use of an enhanced chemiluminescence system (Amersham) with biotinylated secondary antibodies and horseradish peroxidase-avidin.

Localization of Bax protein in 293 cells by using GFP. 293 cells (2×10^6) were transiently transfected with 20 μg of pS65-Bax (ΔTM) or parental pS65T plasmid DNA by a calcium phosphate precipitation method as described above. Approximately 60 h later, the cells were collected and seeded in wells of 24-well plates containing coverslips pretreated with fibronectin for overnight incubation at 37°C. The medium was removed from the wells, and fresh prewarmed (37°C) medium containing 5 to 25 nM MitoTracker (Molecular Probes, Inc.) was added. After incubation for 30 min at 37°C in 95%-5% air-CO_2, the cells were then washed once with PBS (pH 7.3) and fixed in 3.7% paraformaldehyde for 15 min at room temperature. The cells were then rinsed in PBS three times before mounting in Vectashield mounting medium (Vector Laboratories, Inc.) for confocal analysis and photography using an Axiophot photomicroscope (Zeiss, Inc., Oberkochen, Germany).

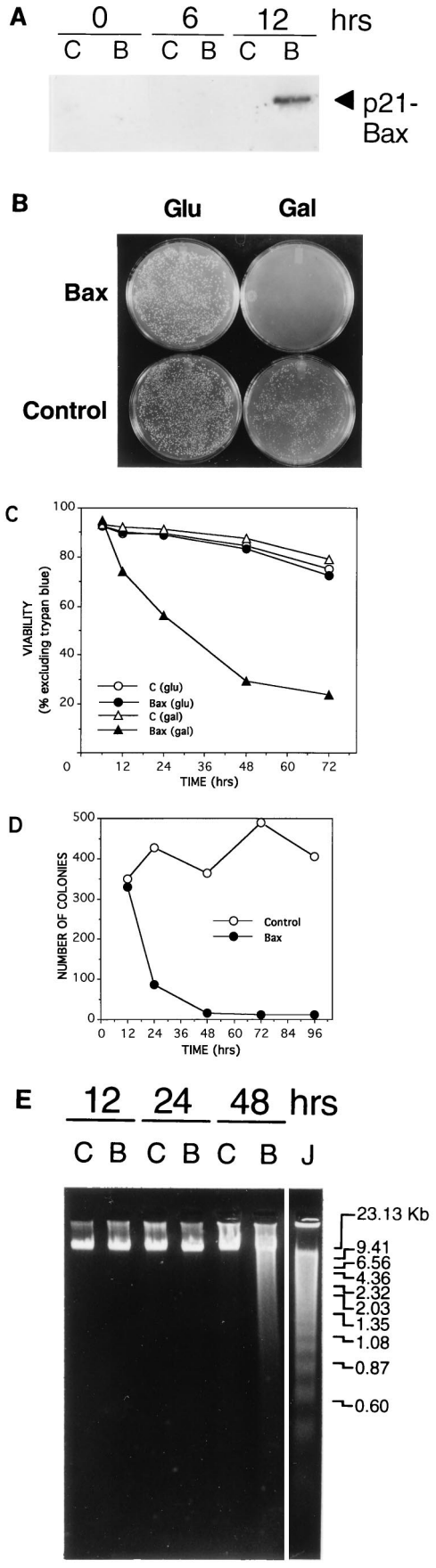
Electron microscopy (EM). Yeast cells containing plasmid YEp51-Bax were cultured for 1 to 2 days in glucose- or galactose-containing medium, washed once with PBS, and then fixed for 30 min in 4 M phosphate buffer containing 3% glutaraldehyde. After being washed twice in 4 M phosphate buffer, cells were embedded in Epon and then postfixed and counterstained in 0.5% osmium tetroxide in 1% uranyl acetate. Ultrathin sections were mounted on grids and imaged with a Hitachi H-600 electron microscope.

RESULTS

Conditional expression of Bax in yeast cells confers a lethal phenotype but does not induce oligonucleosomal DNA degradation. A cDNA encoding the full-length mouse Bax protein was subcloned into the episomal plasmid YEp51, thus placing Bax under the control of a *GAL10* promoter and allowing for conditional expression of this protein when cells are grown in galactose-containing medium. Figure 1A shows an immunoblot analysis of the Bax protein in yeast cells that had been transformed with plasmid YEp51-Bax. Comparisons were made with cells transformed with plasmid YEp51 lacking an insert, as a negative control. When transferred from glucose-based medium, in which the *GAL10* promoter is repressed, to galactose-based medium, the expected 21-kDa Bax protein accumulated to easily detectable levels within 12 h in the cells containing plasmid YEp51-Bax but not in cells harboring the YEp51 control plasmid. The levels of Bax protein produced in yeast cells were comparable to those seen in some mammalian cell lines, when normalized for total protein content (not shown).

Consistent with the galactose-inducible nature of Bax protein production in these cells, plating the YEp51-Bax transformants on semisolid medium that contained galactose resulted in essentially complete inhibition of colony formation, whereas colony formation on glucose-based medium occurred with approximately the same efficiency as observed for control transformants containing plasmid YEp51 without the *bax* cDNA (Fig. 1B). Cotransformation of YEp51-Bax-containing yeast cells with a Bcl-2 expression plasmid, pEG202-Bcl-2 (ΔTM), restored colony formation to about half of control levels (not shown).

To further characterize the effects of Bax on yeast cells, YEp51-Bax- and YEp51-transformed cells were grown in glucose-based medium and then switched to fresh medium containing either galactose or glucose. After various times, cells were recovered from liquid cultures by centrifugation and cell viability was determined on the basis of the ability to exclude trypan blue dye. Figure 1C presents results from a representative experiment, showing a time-dependent decline in the percentage trypan blue-excluding cells in cultures of YEp51-Bax transformants when cultured in galactose-containing medium. The appearance of cells that failed to exclude trypan blue was evident within 12 h after placing cells into galactose-containing medium and generally reached maximal levels at 2 to 3 days. By phase-contrast light microscopy, the dye-excluding and -nonexcluding cells did not appear to be morphologically different in terms of overall size or budding (data not shown). When stripped of their cell wall with Zymolyase, the trypan blue-positive cells essentially disintegrated whereas the dye-excluding cells formed spheroplasts. In contrast to the accumulation of cells which took up trypan blue dye in cultures of YEp51-Bax transformants in galactose-containing medium, cells grown in glucose-based medium remained mostly dye negative (Fig. 1C). The specificity of these results was further confirmed by examination of YEp51 control transformants,



which remained mostly viable regardless of whether cultured in glucose- or galactose-based medium.

To further examine the kinetics of Bax-induced death of yeast cells, YEp51-Bax transformants were cultured for various times in galactose-containing medium to induce Bax expression and then washed and plated on glucose-based semisolid medium in an effort to rescue residual viable cells. Numbers of rescuable colonies were markedly reduced within 1 day of placing these cells into galactose-containing medium, and by 2 to 3 days, very few viable, colony-forming cells remained in cultures (Fig. 1D). In contrast, growth of YEp51 control transformants in galactose-containing medium had no adverse effect on the frequency of viable colony-forming cells. Thus, conditional expression of Bax resulted in an irreversible inhibition of colony formation, consistent with a lethal phenotype. Furthermore, the ability to rescue residual viable cells was dependent on the length of time of Bax induction, with essentially no or very few cells surviving after 2 days. These data therefore provide further confirmation that Bax confers a lethal phenotype in *S. cerevisiae*, as opposed to merely inhibiting cell cycle. In addition, DNA content analysis by flow cytometric analysis of propidium iodide-stained fixed cells failed to reveal accumulation of Bax-expressing cells in any particular phase of the cell cycle (data not shown).

In mammalian cells, apoptotic cell death is characterized by nuclear fragmentation and chromatin condensation, often (but not always) with accompanying cleavage of genomic DNA into fragments representing integer multiples of the internucleosomal distance (~200 bp) (54, 74, 83). Staining Bax-expressing yeast cells with the DNA-binding fluorochrome DAPI failed to reveal evidence of nuclear fragmentation, although loss of nuclear staining occurred at later times after induction of Bax (data not shown). In addition, agarose gel electrophoresis analysis of yeast chromosomal DNA did not reveal the oligonucleosomal pattern of DNA bands typical of apoptotic cells (Fig. 1E). Rather, a diffuse smear of DNA fragments was evident when DNA was prepared from YEp51-Bax transformants after 2 days of culture in galactose-containing medium. At earlier times analyzed after placement of YEp51-Bax cells into galactose medium (12 and 24 h), essentially no difference from control YEp51 transformants was detected in the mobility of

FIG. 1. Conditional expression of Bax confers a lethal phenotype in yeast cells but does not induce oligonucleosomal DNA fragmentation. (A) Strain EGY191 yeast cells were transformed with YEp51 (control; lanes C) or YEp51-Bax (lanes B) plasmids, grown in glucose-based medium at an OD_{600} of ~1.0, then switched to galactose-based medium, and cultured at 30°C for 0, 6, or 12 h. Protein lysates were prepared, and 30-μg aliquots were subjected to SDS-PAGE–immunoblot assay using an anti-Bax antiserum (34). The band shown represents mouse p21-Bax. (B) EGY191 cells transformed with either YEp51 (control) or YEp51-Bax plasmid DNA were spread on either glucose- or galactose-containing plates. Photographs were taken after culturing at 30°C for 4 days. (C) EGY191 cells containing either YEp51 (open symbols) or YEp51-Bax (closed symbols) were grown in glucose-based medium to an OD_{600} of ~1.0, then washed three times, and cultured for 0 to 4 days in either fresh glucose-based (circles) or galactose-based (triangles) medium. Aliquots of cell cultures were removed, and the percentage of cells excluding trypan blue dye was determined by counting a total of ~400 cells. Data are representative of two experiments. (D) Same as panel C except that 10^3 total cells were plated on glucose-based medium. The number of colonies which formed within 4 days of culture at 30°C is indicated for cells containing plasmid YEp51 (open circles) or YEp51-Bax (closed circles). Data are representative of four experiments. (E) Cells containing either YEp51 (lanes C) or YEp51-Bax (lanes B) were cultured for 0, 12, 24, or 48 h in galactose-based medium, and DNA was isolated from 10^7 Zymolyase-prepared spheroplasts. DNA samples were size fractionated by electrophoresis in 2% agarose gels and stained with ethidium bromide. As a control for oligonucleosomal DNA fragmentation, DNA derived from 10^6 Jurkat T cells which had been cultured for 4 h with 20 ng of anti-Fas antibody CH11 (Pharmingen, Inc.) per ml was included in the same gel (lane J).

chromosomal DNA by conventional agarose gel electrophoresis, despite the fact that typically about half of the Bax-expressing cells had lost viability within 24 h after being switched to galactose medium (Fig. 1C and D). In contrast, treating the human T-cell line Jurkat with anti-Fas antibody (a well-known apoptotic stimulus for these cells [71]) for 4 h resulted in DNA degradation in the typical oligonucleosomal pattern (Fig. 1E). Thus, the DNA degradation seen in the setting of Bax-induced death in yeast cells occurs as a relatively late event, probably after loss of plasma membrane integrity, and does not apparently involve endonuclease mechanisms that are the same as or similar to those commonly utilized by many mammalian cells during apoptotic cell death.

To further characterize the cell death process induced by Bax in yeast cells, we undertook EM analysis of cells containing YEp51-Bax after 1 to 2 days of growth in either glucose- or galactose-containing medium. EM analysis of yeast cells growing in glucose-containing medium, in which Bax expression is not induced, showed typical morphology for normal *S. cerevisiae*, with a central vacuole, several mitochondria, and homogeneous nuclear chromatin except for the nucleolar region (Fig. 2A). In contrast, cells grown in galactose-containing medium developed a variety of morphological abnormalities, which presumably reflected a continuum of severity. For example, some cells were largely normal but contained increased numbers of cytosolic vacuoles (Fig. 2B). Cells further into the degenerative process were characterized by dissolution of the nucleus, enlargement of cytoplasmic vacuoles, and evidence of mitochondrial swelling (Fig. 2C). Finally, cells in the terminal stages of the cell death process had undergone nearly complete autophagy, with absence of the nucleus and most cytosolic organelles, and contained only the remnants of what appeared to be ruptured mitochondria. The cell wall was evidently intact and abutted by electron-dense material that appeared to be largely contained within membranous structures. Taken together, therefore, the data presented in Fig. 1 and 2 indicate that the cell death induced by Bax in yeast cells does not occur by mechanisms consistent with apoptosis, as currently recognized in mammalian and metazoan species.

Membrane targeting of Bax protein is required for its lethal phenotype in yeast cells. Like most members of the Bcl-2 protein family, the Bax protein contains a stretch of hydrophobic amino acids near its C terminus that meets computer predictions for a TM domain (55). To determine the role of this membrane anchoring sequence for the function of the Bax protein in yeast cells, we prepared expression plasmids that contained cDNAs encoding either the full-length Bax protein or a truncation mutant of Bax lacking the C-terminal TM domain [Bax (ΔTM)] under the control of a constitutive *ADH* promoter (Fig. 3A, top). These and all other Bax mutant proteins subsequently described in this report were expressed as fusion proteins which contained an N-terminal LexA DNA binding domain lacking a nuclear localization signal sequence, similar to the approach used in previous studies (4, 19, 61). The LexA sequences served as an epitope tag for monitoring expression of proteins and also were intended to promote the stability of truncated proteins when expressed in *S. cerevisiae* (see below).

Plasmids encoding the full-length Bax or Bax (ΔTM) protein and carrying a *HIS3* marker were then transformed into yeast cells, and the cells were cultured on histidine-deficient medium. Four days later, the relative numbers of colonies of viable cells were enumerated, and the data were normalized relative to transformations performed with either the same parental plasmid pEG202 encoding the LexA domain alone or a LexA-lamin fusion protein which served as a negative control. As shown in Fig. 3A, the full-length Bax plasmid suppressed colony formation by >99% in this assay. In contrast, the Bax (ΔTM) plasmid had no inhibitory effect. The inhibition of colony formation by the full-length Bax expression plasmid did not appear to be due to nonspecific toxicity caused by the *bax* cDNA sequences, because essentially no suppression of colony formation occurred when cells were transformed with the same plasmid in which the *bax* cDNA has been subcloned in reversed (antisense) orientation (Fig. 3A). Moreover, cotransfection of plasmid pJG4-5-Bcl-2 (ΔTM), which produces a Bcl-2 fusion protein, restored colony formation to ~50% of control levels (not shown), as reported previously (61).

This experiment suggested that the TM domain of Bax is required for its lethal phenotype in yeast cells. However, it was also possible that the TM domain was nonspecifically toxic when overexpressed in yeast cells, perhaps by indiscriminately impairing the function of other membrane proteins. To explore this possibility, an expression plasmid that encoded a N-LexA fusion protein containing only the TM domain of Bax was prepared. When expressed in yeast cells, this LexA-TM fusion protein had no inhibitory effect on colony formation, suggesting that the TM domain of Bax is not nonspecifically toxic (Fig. 3A).

Since these data suggested that membrane targeting is critical for the lethal phenotype of Bax in yeast cells, we next attempted to restore cytotoxic function to the Bax (ΔTM) protein by fusing this portion of Bax (amino acids 1 to 171) with a heterologous TM domain derived from another protein. The TM domain of the yeast outer-membrane mitochondrial protein Mas70p was chosen for these experiments on the basis of previous reports showing that this sequence can restore optimal function to C-truncation mutants of Bcl-2 that lack a TM domain (52) and because of evidence that most Bcl-2 family proteins are primarily associated with mitochondria (14, 24, 36, 38, 48, 84). When transformed into yeast cells, expression plasmids encoding the Bax-Mas70p fusion protein suppressed colony formation with efficiencies comparable to that of the wild-type Bax protein (Fig. 3A).

Immunoblot assays were performed to verify that the various expression plasmids described above produced the expected fusion proteins. However, because no viable colonies of cells could be recovered from cells transformed with plasmids encoding the full-length Bax and Bax-Mas70p proteins, it was necessary to cotransform cells with a Bcl-2 expression plasmid. For these immunoblot experiments, we took advantage of the N-terminal LexA tag sequences for detection of proteins by using an anti-LexA antiserum (gift of E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa.). All yeast cell lysates were normalized for total protein content prior to analysis. As shown in Fig. 2B, the Bax (ΔTM) protein was produced at levels about three times higher than those of the full-length Bax protein, thus excluding insufficient production of this C-truncation mutant as an explanation for its failure to suppress colony formation. Similarly, the LexA-TM fusion protein was produced at levels about five times higher than those of the wild-type Bax protein, indicating that the inability of the TM domain of Bax by itself to kill yeast cells was not an artifact of poor levels of expression. The Bax-Mas70p fusion protein was produced at levels comparable to those of the wild-type Bax protein (Fig. 3B).

Bax is targeted to mitochondria in yeast cells. Because membrane anchoring was critical for the lethal phenotype of Bax in yeast cells, we compared the intracellular locations of the Bax, Bax (ΔTM), and Bax-Mas70p proteins by immunofluorescence microscopy, using a mouse monoclonal anti-Bax antibody in conjunction with a rhodamine-labeled secondary an-

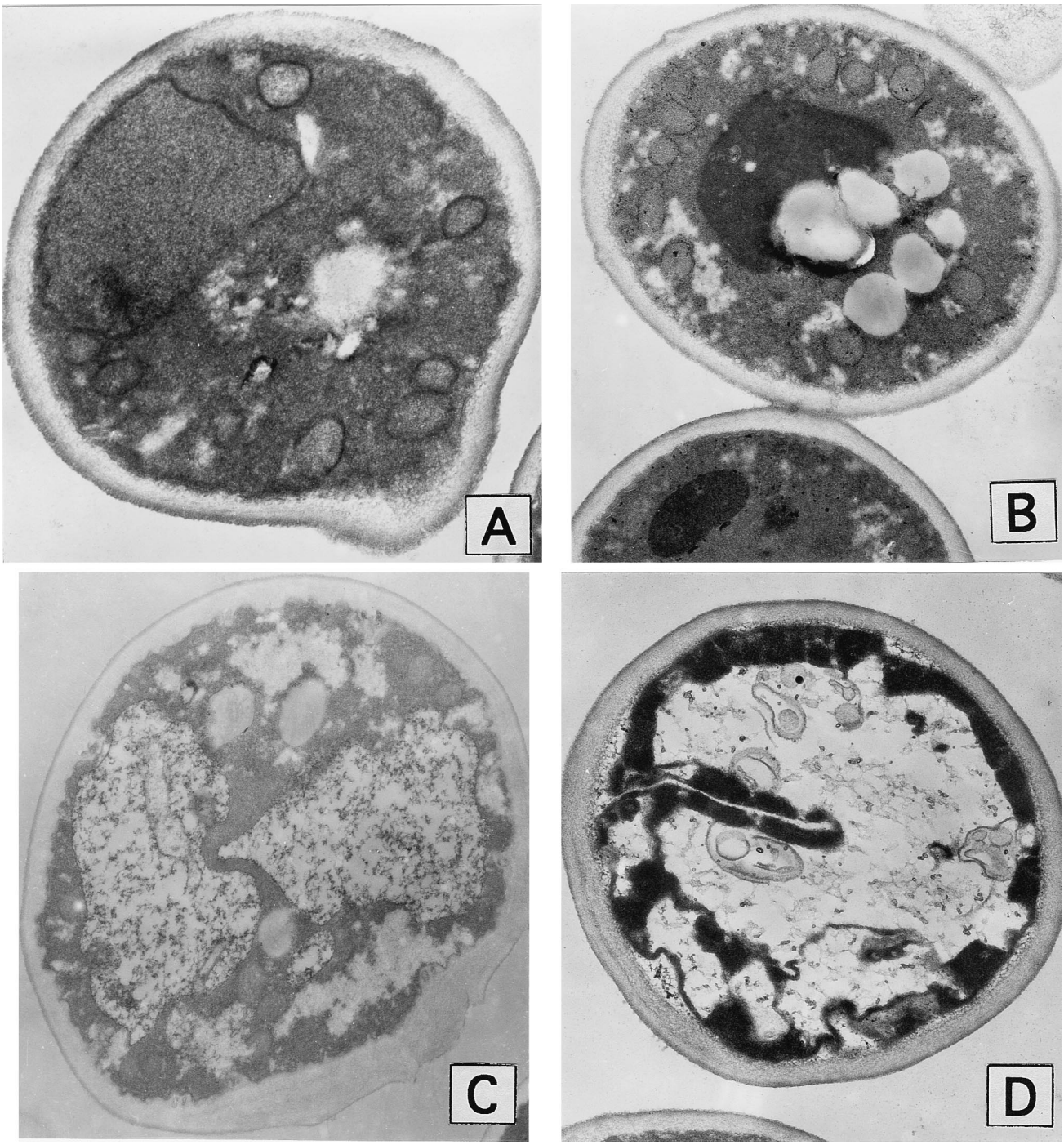


FIG. 2. EM analysis of Bax-mediated cell death in yeast cells. EGY48 cells containing YEp51-Bax were cultured in glucose-containing (A) or galactose-containing (B to D) medium for 1 to 2 days. Cell morphology was then analyzed by EM (magnification, ×~45,000). The cells shown in panels B to D are representative of the spectrum of changes that were seen in cells expressing Bax, from least to most affected.

tibody. As shown in Fig. 4, the Bax and Bax-Mas70p proteins were located in the cytosol of yeast cells in a punctate immunofluorescence pattern, suggestive of organellar association. Costaining of these cells with a rabbit antiserum specific for the yeast mitochondrial outer-membrane protein OM14 (using a fluorescein isothiocyanate-labeled secondary antibody) indicated that these organelles with which Bax was associated were probably mitochondria. Consistent with this idea, when these same cells were incubated with the DNA-binding fluorochrome DAPI, which labels both chromosomal and mitochondrial DNA, the Bax immunofluorescence colocalized with the mitochondrial DNA staining but not nuclear genomic DNA staining (Fig. 4). In contrast to the wild-type Bax and Bax-Mas70p proteins, the immunofluorescence pattern obtained for the Bax (ΔTM) protein revealed mostly diffuse cytosolic and nuclear staining which lacked any obvious colocalization

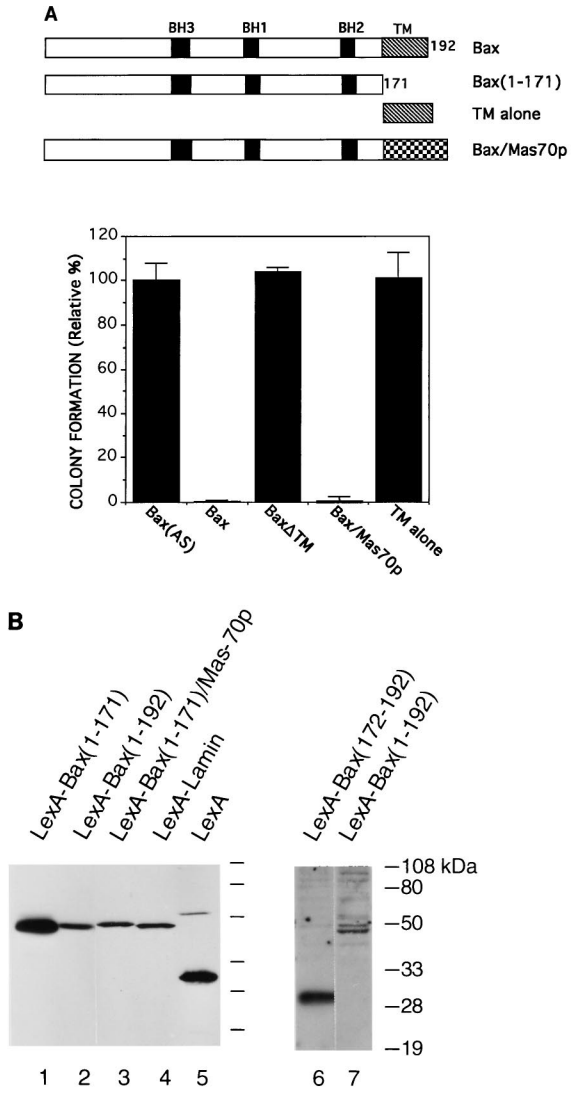


FIG. 3. Targeting to intracellular membranes is essential for the lethal phenotype of Bax in yeast cells. (A) Bax, Bax (ΔTM), TM, and Bax-Mas70p were expressed as fusion proteins containing an NH_2-terminal LexA domain under the control of an *ADH* promoter in plasmid pEG202, which contains a *HIS3* gene. After transformation into yeast cells and plating on histidine-deficient media for 4 days at 30°C, the colonies were counted on the plates. The data shown were obtained from three independent experiments performed on different days with different preparations of plasmid DNA (mean ± standard deviation) and are expressed as percentages relative to cells transformed with either pEG202 lacking a cDNA insert or pEG202-lamin, which were used as negative controls and whose level was arbitrarily set at 100%. (B) The relative levels of the LexA fusion proteins Bax, Bax (ΔTM), TM, and Bax-Mas70p described above were examined by immunoblot analysis of 20 μg of total protein lysate, using an anti-LexA antibody. All cells had been cotransformed with plasmid pJG4-5-Bcl-2 (ΔTM) to suppress the lethal effects of full-length Bax and Bax-Mas70p.

with OM14 or DAPI. Taken together, these data strongly suggest that the Bax protein is associated with mitochondria when expressed in yeast cells, whereas the Bax (ΔTM) protein, which lacks cytotoxic function, fails to associate with these organelles.

A well-conserved four-amino-acid sequence in the BH3 domain of Bax is required for homodimerization. Recently we demonstrated that the BH3 domain of Bax is absolutely required for homodimerization with Bax and for heterodimerization with Bcl-2, on the basis of the results of yeast two-hybrid assays and in vitro protein interaction assays (88). Though binding constants (K_d) have not been determined to date, Bax-Bax homodimerization appears to occur in the complete absence of the BH1, BH2, and N-terminal (residues 1 to 58) domains with efficiencies comparable to that of the full-length Bax protein (88). Within the BH3 domain of Bax and its proapoptotic homologs Bak and Bik is a conserved four-amino-acid sequence, IGDE (residues 66 to 69 in the mouse Bax protein). We therefore prepared two-hybrid expression plasmids encoding a deletion mutant of Bax lacking these four amino acids, Bax (ΔIGDE), fused with either an N-terminal LexA DNA binding domain or a B42 TA domain and then compared the wild-type Bax and Bax (ΔIGDE) proteins with regard to ability to homodimerize, using *lacZ* and *LEU2* reporter genes under the control of *lexA* operators (13). Both the Bax and Bax (ΔIGDE) proteins were expressed without their TM domains to prevent problems with nuclear targeting of the hybrid proteins and to avoid Bax-mediated cytotoxicity. Immunoblotting demonstrated comparable levels of expression of the wild-type and ΔIGDE Bax proteins (not shown).

With either β-galactosidase filter assays (Fig. 5) or growth on leucine-deficient media (not shown) as endpoints, the wild-type Bax protein formed strong two-hybrid interactions with itself when cells were plated on galactose-based medium, which induces expression of the TA domain-containing partner protein in these assays (under control of *GAL1* promoter), but not when plated on glucose-based medium. In contrast, the Bax (ΔIGDE) protein failed to interact with itself at levels substantially above background, as defined by comparisons with two-hybrid results of assays in which Bax (ΔIGDE) was paired with either R-Ras or Fas (cytosolic domain) as a negative control (Fig. 5). The Bax (ΔIGDE) protein also failed to interact with Bcl-2 in two-hybrid assays, whereas the wild-type Bax protein formed strong interactions with Bcl-2 (Fig. 5).

Bax homodimerization is required for lethal phenotype in yeast cells. To compare the functions of the wild-type and ΔIGDE mutants of Bax in yeast cells, we prepared expression plasmids containing a *HIS3* marker that encoded the Bax and Bax (ΔIGDE) proteins with TM domains under the control of an *ADH* promoter. When transformed into yeast cells and plated on histidine-deficient medium, the full-length, wild-type Bax protein suppressed colony formation by >99% (Fig. 6A). In contrast, the Bax (ΔIGDE) protein was completely inactive in this assay, allowing for colony formation at frequencies comparable to those of negative control plasmids. Immunoblot analysis demonstrated that the Bax (ΔIGDE) protein was produced at levels ~3-fold higher than those of wild-type Bax (Fig. 6B), excluding poor levels of expression of this mutant protein as a trivial explanation for the lack of function in yeast cells. These results therefore suggest that for Bax to manifest its lethal phenotype in yeast cells, it must be able to homodimerize. The findings also provide further evidence that the cytotoxic effects of Bax in yeast cells cannot be attributed to a nonspecific mechanism, since deletion of just four amino acids completely abolished the activity of this protein.

Though homodimerization of Bax appeared to be important for its function in yeast cells, we wished to explore whether mutants of Bax that lacked other conserved domains but which remained capable of homodimerizing also retained their lethal function when expressed in yeast cells. For this purpose, Bax (ΔBH1), Bax (ΔBH2), and Bax (ΔN) mutant proteins (with their TM domains) were tested for suppression of colony formation in transformation assays as described above. As shown in Fig. 6A, the Bax (ΔBH1), Bax (ΔBH2), and Bax (ΔN) proteins retained ~50, ~60, and ~80%, respectively, of their death-promoting function in yeast cells. Immunoblot analysis confirmed that all of these proteins were expressed at levels at

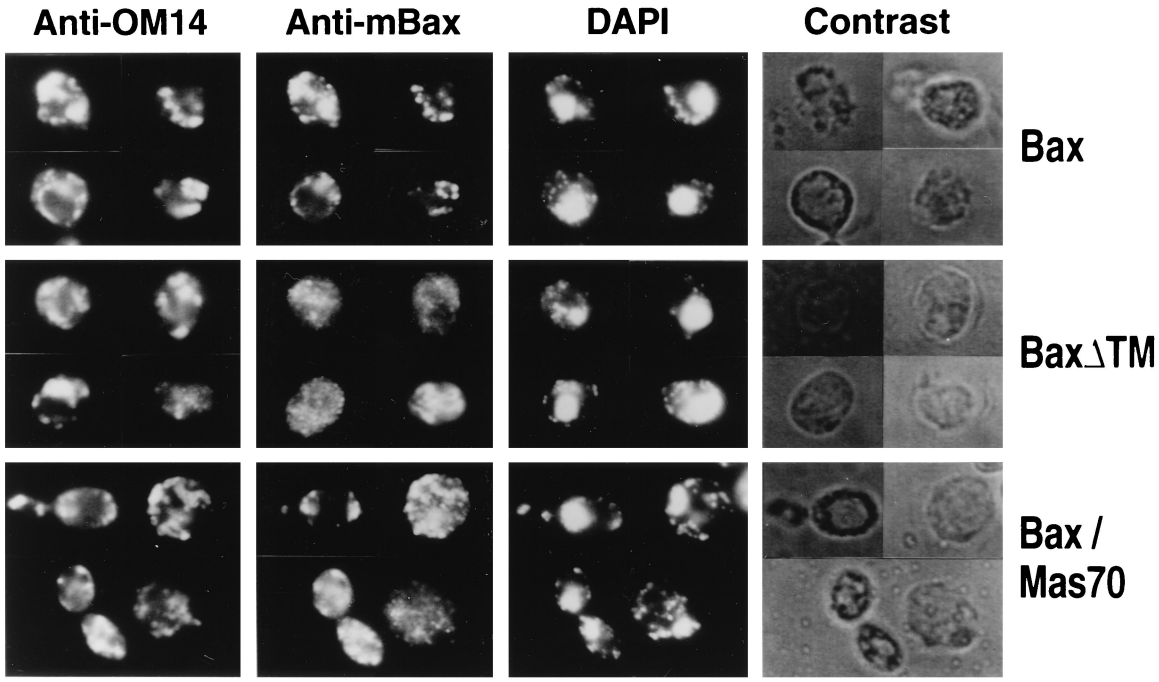


FIG. 4. Mitochondrial localization of Bax detected by indirect immunofluorescence. Bax, Bax (ΔTM), and Bax-Mas70p were expressed as LexA fusion proteins in yeast cells. The cells were also cotransformed with the expression plasmid pJG4-5-Bcl-2 (ΔTM) to maintain cell survival. The primary antibodies used were rat IgM monoclonal anti-Bax and mouse IgG monoclonal anti-OM14. The secondary antibodies were rhodamine isothiocyanate-goat anti-rat IgM and fluorescein isothiocyanate-donkey anti-mouse IgG, respectively. The cells were also stained with DAPI (see Materials and Methods for details). The right-most panel shows phase-contrast photomicrographs of the same cells.

least equal to or (in most cases) greater than that of the wild-type Bax protein (Fig. 6B). Thus, while these other domains within the Bax protein may contribute to the optimal function of this protein, none is absolutely required for Bax-mediated killing of yeast cells.

The Bax (ΔIGDE) protein is defective in promoting apoptosis in mammalian cells. Because versions of Bax that lacked a TM domain or that were incapable of homodimerizing represented loss-of-function mutants in yeast cells, we contrasted the bioactivities of these proteins with that of the wild-type Bax protein in mammalian cells. For these experiments, we constructed expression plasmids that encoded the Bax, Bax (ΔTM), and Bax (ΔIGDE) proteins with NH_2-terminal HA epitope tags under the control of a strong constitutive viral promoter (cytomegalovirus immediate-early region). These plasmids or the empty vector (control) were cotransfected with the β-galactosidase-producing plasmid pCMV-β-Gal into Rat-1 fibroblasts, and 2 days later the relative numbers of β-galactosidase-expressing (blue) cells were enumerated. In this assay, Rat-1 cells which die by apoptosis round up and detach from dishes, making the relative number of adherent blue cells a good indicator of Bax protein function. This same transient transfection assay has been used previously for studies of Bak, Bik, and other Bcl-2 family proteins, as well for studies of several types of proapoptotic proteins (6, 8, 9, 45, 78).

As shown in Fig. 7A, very few blue cells were detected in monolayer cultures of Rat-1 cells when transfected with the wild-type Bax expression plasmid, and about half of these β-galactosidase-positive cells had obvious morphological features of apoptosis, such as rounded shape and shrunken size as well as nuclear fragmentation, which was confirmed by propidium iodide staining (not shown). Bax (ΔTM) was only slightly less effective than wild-type Bax in this assay (Fig. 7A). Thus, while the presence of a membrane-anchoring domain is crucial for the prodeath function of Bax in yeast cells, it is not required for function in mammalian cells. In contrast, in cultures of Rat-1 cells transfected with the Bax (ΔIGDE) plasmid, blue cells were present at levels comparable to or slightly higher than those of control plasmid-transfected cells, and <10% of these had morphological features suggestive of apoptosis. Similar to the loss of function seen in yeast cells, therefore, this ho-

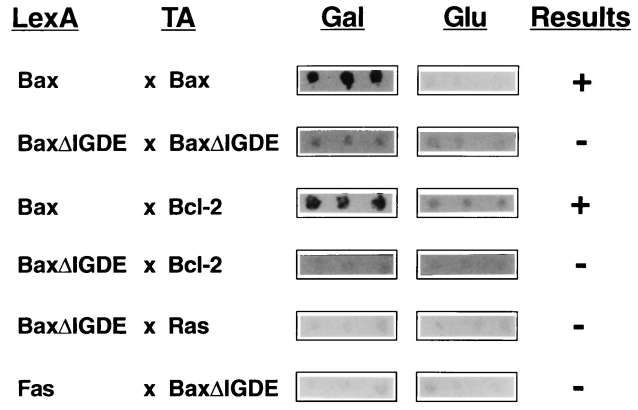


FIG. 5. Two-hybrid comparison of dimerization by Bax and Bax (ΔIGDE). Bax, Bax (ΔIGDE), Bcl-2, and other proteins tested in this experiment were expressed in EGY191 as fusion proteins with either NH_2-terminal LexA DNA binding domain from pEG202 or TA domain from plasmid pJG4-5, as indicated. The data shown were obtained from β-galactosidase filter assays. Three independent transformants were plated on either galactose- or glucose-containing medium.

modimerization-defective mutant of Bax failed to promote apoptosis in mammalian cells.

Similar results were obtained in 293 kidney epithelial cells. Because the efficiency of transfection was much higher for these cells (~80%) than for Rat-1 cells, 293 cells were examined directly by DAPI staining without β-galactosidase as a marker plasmid. Approximately one-quarter of the 293 cells in cultures transfected with either the Bax or Bax (ΔTM) expression plasmid developed the nuclear fragmentation typical of apoptosis, compared with <5% of the cells transfected with the Neo control or Bax (ΔIGDE)-producing plasmids (Fig. 7B and C).

To confirm that the Bax (ΔIGDE) protein was stable when produced in mammalian cells, 293 human kidney epithelial cells were transiently transfected with the same Bax and Bax (ΔIGDE) expression plasmids used for the Rat-1 transfection assay. Immunoblot analysis performed with a monoclonal antibody directed against the HA epitope tags demonstrated comparable levels of the HA-Bax and HA-Bax (ΔIGDE) proteins, suggesting that the failure of the Bax (ΔIGDE) protein to promote cell death was not attributable to protein instability (Fig. 7D, lanes 1 and 3). The Bax (ΔTM) protein was also produced at similar levels (Fig. 7D, lane 2).

The abilities of the mouse HA-Bax and HA-Bax (ΔIGDE) proteins to coimmunoprecipitate with the endogenous human Bax protein in 293 were then compared. For these experiments, immunoprecipitations were performed with anti-HA antibody and the resulting immune complexes were subjected to SDS-PAGE–immunoblot analysis using an antipeptide antiserum specific for the human Bax protein (33). As shown in Fig. 7E (lanes 1 and 3), human Bax protein coimmunoprecipitated with the wild-type mouse HA-Bax protein but not with the HA-Bax (ΔIGDE) protein. The endogenous wild-type human Bax protein also coimmunoprecipitated with the HA-Bax (ΔTM) protein (Fig. 7E, lane 2). Incubating the blot with the anti-HA monoclonal antibody confirmed that equivalent amounts of the HA-Bax and HA-Bax (ΔIGDE) proteins had been immunoprecipitated (not shown). These findings thus confirm in mammalian cells the results of the yeast two-hybrid experiments which suggested that the Bax (ΔIGDE) protein was incapable of homodimerizing.

A TM domain is not required for targeting of Bax to mitochondria in mammalian cells. In yeast cells, the removal of the TM domain from Bax resulted in loss of targeting to mitochondria and loss of function. In contrast, the Bax (ΔTM) protein retained nearly full activity as an inducer of apoptosis in Rat-1 cells. To explore the location of Bax (ΔTM) in mammalian cells, this protein was expressed in 293 cells as a fusion protein with the GFP(S65T) protein, a mutant form of GFP that has stronger fluorescence properties than the wild-type protein (21). Cells were also stained with a mitochondrion-specific rhodamine-like dye to visualize these organelles. Examination of these GFP(S65T)-Bax (ΔTM)-expressing cells by fluorescence confocal microscopy demonstrated the presence of the GFP-Bax protein in the cytosol in a punctate pattern, suggestive of organelle association (Fig. 8a). A similar pattern was evident for the mitochondrion-specific stain as well (Fig. 8b). Two-color analysis revealed colocalization of the GFP(S65T)-Bax (ΔTM) protein and mitochondria (Fig. 8c). In contrast, when the GFP(S65T) protein was expressed in 293 cells as a control, the only green fluorescence observed was located diffusely throughout the cytosol and did not colocalize with mitochondria (Fig. 8d to f). Unlike in yeast cells, therefore, a TM domain is not required for targeting of Bax to mitochondria in mammalian cells, presumably because mammalian cells contain endogenous Bax or other proteins with which the Bax (ΔTM) protein can interact.

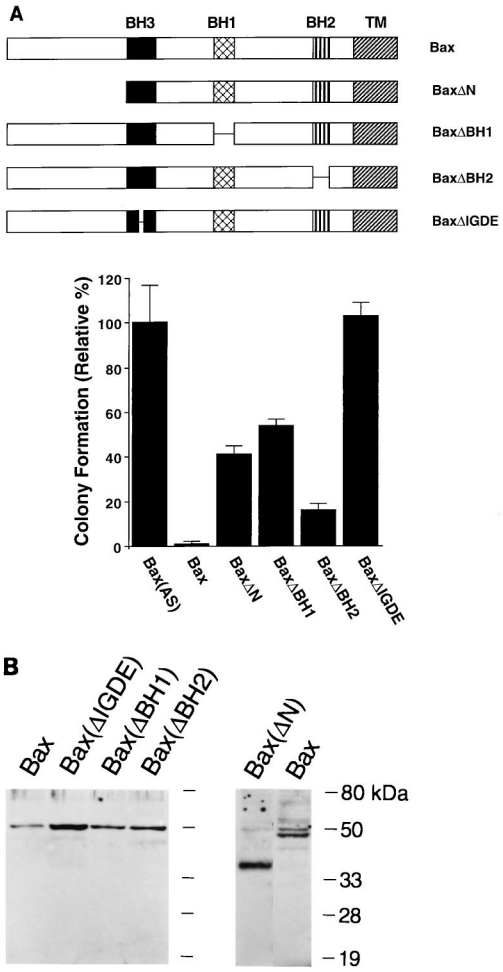


FIG. 6. Evaluation of cytotoxic function of Bax (ΔIGDE) and other mutants in yeast cells. EGY191 cells were transformed with plasmids encoding the full-length wild-type Bax protein or various Bax mutants (expressed as LexA fusions) and plated onto histidine-deficient medium. Colonies were counted after incubation at 30°C for 4 days. The data shown were obtained from three experiments (mean ± standard deviation). Bax(AS) represents cells transformed with the same pEG202 plasmid containing the *bax* cDNA subcloned in antisense orientation. (B) The relative levels of the LexA fusion Bax and Bax mutant proteins were determined by immunoblotting (30 μg) using anti-LexA antibody.

DISCUSSION

In yeast cells, the cell death process induced by Bax did not resemble apoptosis, inasmuch as no evidence of nuclear fragmentation and chromatin condensation was evident by EM and in that the DNA degradation which occurred in Bax-expressing yeast cells was a late event that did not involve the oligonucleosomal degradation pattern which classically occurs in mammalian cells when induced to undergo apoptosis. Though DNA ladders that can be detected by conventional agarose gel electrophoresis are not necessarily seen in apoptotic cells (54, 74, 83), the morphological changes in nuclear shape and chromatin condensation are essentially universal features of apoptosis in mammalian cells but were not found in Bax-expressing *S. cerevisiae*. Instead, the cell death process induced by Bax in yeast cells resembled autophagy, with dissolution of the internal organelles and vacuolarization.

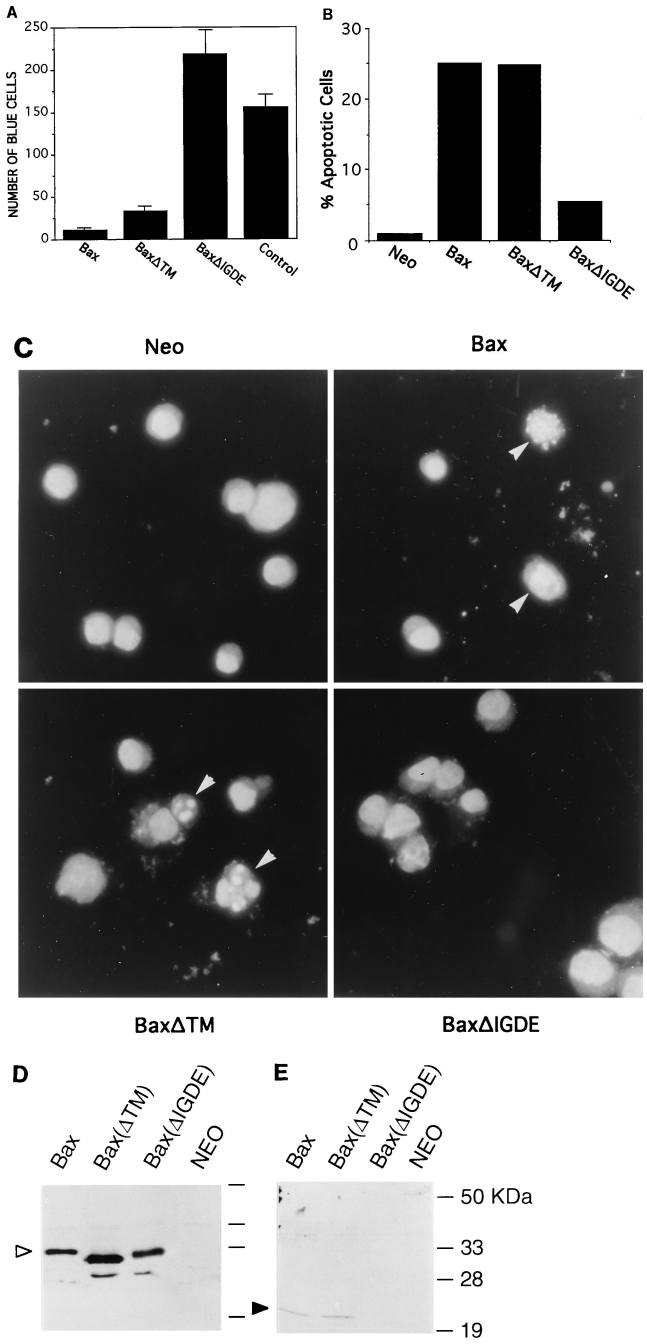


FIG. 7. Effects of Bax, Bax (ΔIGDE) and Bax (ΔTM) on apoptosis in Rat-1 and 293 cells. (A) Bax, Bax (ΔIGDE), and Bax (ΔTM) were subcloned into plasmid pHA-Shin for expression as HA epitope-tagged proteins. Rat-1 cells were cotransfected with pHA-Shin (control), pHA-Shin-Bax, pHA-Shin-Bax (ΔIGDE), or pHA-Shin-Bax (ΔTM) and the β-galactosidase reporter plasmid, pCMV-β-gal. Cells were fixed and stained with X-Gal 24 h after transfection, and the number of blue cells (β-galactosidase positive) was counted by microscopic examination (mean ± standard deviation for triplicate transformations). (B) 293 cells were transfected with 7 μg of pcDNA3-HA-Bax, pcDNA3-HA-Bax (ΔTM), pcDNA3-HA-Bax (ΔIGDE), or parental pcDNA3-HA plasmid DNA by a calcium phosphate precipitation method. Approximately 36 h later, the floating and adherent cells were pooled and stained with DAPI, and the percentage of cells with nuclear fragmentation was determined (data representative of two experiments). (C) Representative photographs of the transfected 293 cells used for panel B, illustrating the nuclear fragmentation typical of apoptotic DAPI-stained cells compared with normal cells with intact nuclei. (D and E) 293 cells were transiently transfected with 20 μg of pcDNA3-HA-Bax, pcDNA3-HA-Bax (ΔTM), pcDNA3-HA-Bax (ΔIGDE), or pcDNA3-HA (pShin-HA) parental plasmid DNA, and ~60 h later, cell lysates were prepared. After normalization for total protein content, lysates were either used directly (30 μg; D) or immunoprecipitated (300 μg) with anti-HA monoclonal antibody (E) and then analyzed by SDS-PAGE–immunoblotting using either anti-HA (D) or anti-human Bax (E) antibody. The open arrowhead indicates positions of three-HA epitope-tagged Bax, Bax (ΔTM), and Bax (ΔIGDE) proteins. The closed arrowhead indicates human Bax protein. Detection was by an enhanced chemiluminescence method.

It should be noted, however, that not all programmed cell deaths (PCDs) occur with the classical features of apoptosis in animal and plant species (64). For example, the death of intersegmental muscle cells that occurs near the end of metamorphosis in the moth *Manduca sexta* involves the cell shrinkage typically seen during apoptosis, but plasma membrane blebbing, production of apoptotic bodies, and nuclear fragmentation do not occur (64). Furthermore, the only chromatin condensation seen in these PCDs occurs in a punctate, focal pattern; in apoptotic cells, by contrast, margination of condensed chromatin against the nuclear envelope is typically observed. The PCD of muscle cells in the moth also does not involve internucleosomal DNA cleavage (64). Similarly, PCD of the stalk cells of sorocarps, multicellular fungus-like structures formed by the unicellular protist *Dictyostelium discoideum* when grown under conditions of nutrient insufficiency or overcrowding, involves cytoplasmic vacuolarization and cytosolic condensation similar to classical apoptosis but membrane blebbing is absent, possibly because of the presence of a cell wall. Nuclear fragmentation is also not seen during PCD of these stalk cells, but multifocal chromatin condensation which is distinctly different from the chromatin margination seen in classical apoptosis is detectable by EM. These PCDs also occur in the absence of DNA degradation that can be detected by either routine agarose gel electrophoresis or pulsed-field gel electrophoresis (10). Likewise, the PCD response induced by pathogens in tobacco (the hypersensitive response) is associated with induction of endonuclease activities and genomic DNA digestion, but the DNA fragmentation does not involve the oligonucleosomal pattern of DNA cleavage or nuclear morphological changes often seen in apoptosis (44). These morphological and biochemical differences in PCD in various plant and animal species argue that at least the distal portions of the cell death pathways have diverged during evolution. It therefore remains to be established whether more proximal elements of these cell death pathways are conserved. For example, while a family of cysteine proteases has been implicated recently as major effectors of apoptosis in animal species (reviewed in reference 40), to date there is no evidence that plant species rely on similar proteins for execution of their PCDs. Regardless, from comparisons of PCD in various animal and plant species, it is clear that the absence of oligonucleosomal DNA fragmentation and nuclear fragmentation does not necessarily preclude the possibility that the mechanism by which Bax induces cell death in yeast cells is physiologically relevant to mammalian cells, nor does it prove it.

Related to this issue of whether the cell death process induced by Bax in yeast cells is physiologically meaningful for mammalian cells is the observation that while Bcl-2 can block cell death induced by numerous stimuli that result in apoptosis, under some circumstances overexpression of Bcl-2 has also been shown to prevent cell deaths that were clearly necrotic rather than apoptotic in their morphological and biochemical characteristics (28, 29, 66, 69). For example, necrotic cell death induced in neuronal cell lines in vitro by agents that deplete intracellular glutathione is blockable by Bcl-2, suggesting that Bcl-2 family proteins may regulate a cellular process that leads to apoptosis in some cases but necrosis in others (28, 29).

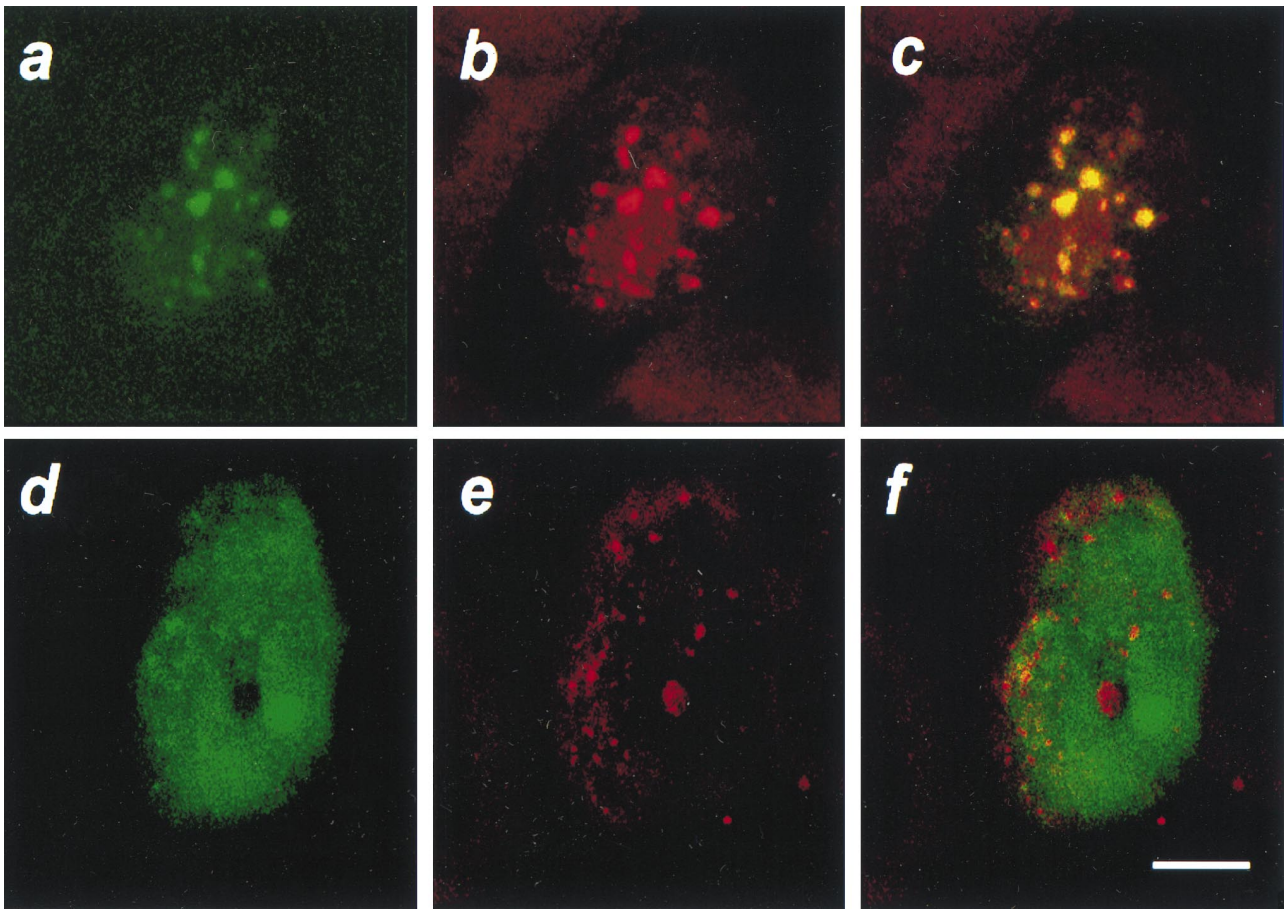


FIG. 8. GFP-Bax (ΔTM) protein colocalizes with mitochondria in 293 cells. 293 cells were transiently transfected with pS65T-Bax (ΔTM) (a to c) or parental pS65T (d to f) plasmid DNA. After ~60 h, the cells were incubated with MitoTracker dye and analyzed by fluorescence confocal microscopy using appropriate filters for visualization of green (A) or red (B) fluorescence, or both (C), resulting from the GFP(S65T) and MitoTracker molecules. Data are representative of the majority of doubly stained cells.

Similarly, Bcl-2 and Bcl-X_L can prevent cell death induced in vitro by hypoxia, an insult typically considered to be a classical inducer of necrotic cell death (15, 27, 67). Though much of the cell death that occurs in the setting of hypoxia in vitro may be due to apoptosis, some is clearly necrotic in its morphological characteristics and is blockable by Bcl-2 and Bcl-X_L (66). In experiments involving transgenic mice which overexpress Bcl-2 in central nervous system neurons, Bcl-2 has also been shown to significantly reduce hypoxia-induced cell death in vivo in models of stroke, a situation that results in both apoptotic and necrotic cell deaths (41). Finally, Bcl-2 can prevent necrotic cell death induced by viral infection (69).

Taken together, therefore, these data indicate that Bcl-2 family proteins can regulate cell death pathways that do not necessarily culminate in apoptosis. Furthermore, elements of this cell death mechanism may be evolutionarily conserved even as far back as yeast species, since the Bax protein can induce death in the budding yeast *S. cerevisiae* and Bcl-2 can rescue these cells from the cytotoxic effects of Bax. Bcl-2 has also been shown to rescue mutant yeast strains with defects in superoxide dismutase from the lethal effects of growth under aerobic conditions (29), providing further evidence that at least portions of the cell death mechanism regulated by Bcl-2 family proteins may be operative in yeast cells. Moreover, we have recently observed that the proapoptotic proteins Bax and Bak have a lethal phenotype when expressed in the fission yeast *Schizosaccharomyces pombe* and that Bcl-X_L but not Bcl-X_S can rescue these cells from Bax- and Bak-mediated death (unpublished data). Thus, whatever the cell death mechanism employed by Bax and other Bcl-2 family proteins in *S. cerevisiae*, it appears to be functional in *S. pombe* as well. It remains to be determined whether this potentially conserved function derives from the recently hypothesized function of Bcl-2 family proteins as pore-forming molecules (50) rather than through their functional or physical interactions with other conserved proteins in yeast cells.

Cell suicide behaviors have been observed in a variety of 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 references 68, 81, and 85). For example, some strains of *Escherichia coli* will activate the expression of genes that trigger cell death when infected with bacteriophages. Moreover, many other examples of cell death exist in unicellular bacterial species that could reasonably be classified as suicide or programmed in that the cell is an active participant in its own demise and the death of individual cells affords a survival advantage to other cells that derive from common antecedents, including the cell deaths associated with (i) restriction-modification systems involving restriction endonucleases and counteracting methylases, (ii) cell suicide following DNA damage induced by irradiation or chemicals in bacteria

carrying colicin toxin genes, (iii) the sacrifice of mother cells during sporulation of bacilli, and (iv) the death and subsequent cannibalization of cells during fruiting body formation by myxobacteria (reviewed in references 68, 81, and 85). Moreover, cell death occurring with the classical features of apoptosis has also been reported for some eukaryotic unicellular organisms, such as *Trypanosoma* species, which have been shown to undergo apoptotic cell death in response to high cell densities or insufficient nutrients (1, 79). Finally, the discovery that some PCD-regulatory genes have homologs in both plants and animals (such as the antiapoptotic gene *dad-1*) lends further weight to the argument that some portions of the cell death machinery may have evolved originally in unicellular organisms, before the divergence of the plant and animal kingdoms (2). Though several potential evolutionary advantages to having cell suicide mechanisms even in unicellular organisms can be imagined, defenses against viruses represent a particularly compelling argument (reviewed in references 76, 77, and 81). Thus, when a virus infects a yeast culture, for example, one mechanism for limiting viral spread and thereby preserving the genomes of the progeny of these unicellular organisms would be for the virus-infected cell to commit suicide before viral replication can occur. Elements of this defense mechanism then could have been transferred to and embellished upon during evolution in metazoans, where it again would have served the organism well when confronted with viruses.

The finding that a membrane-anchoring domain is essential for Bax function in yeast cells but expendable in mammalian cells raises the possibility of differences in the way this death-promoting protein functions in these two types of cells. It is more likely, however, that in mammalian cells, Bax does not require a TM domain for targeting to appropriate intracellular membranes because of its ability to dimerize with endogenous Bax and other Bcl-2 family proteins that are already integrated into the outer mitochondrial membrane because of their TM domains. In contrast to Rat-1 and 293 cells, we have observed that removal of the TM from Bax abrogates its proapoptotic function in some types of cells (unpublished observations). Thus, differences between Bax (ΔTM) function in Rat-1 and 293 cells and in some other cell lines raise the possibility that the efficiency with which Bax (ΔTM) targets to mitochondria or other appropriate membrane compartments differs among cell types. By analogy, it has been observed that the relative importance of a TM domain in Bcl-2 varies widely among various cell lines for its antiapoptotic function, with some cells displaying essentially no dependence, others showing partial dependence, and still others exhibiting complete dependence on a membrane-targeting domain (5, 23, 24, 52, 72). Taking these data together with the data presented here, therefore, we speculate that either yeast cells lack homologous proteins to which Bax (ΔTM) mutants can dimerize or the interaction of Bax (ΔTM) with these yeast proteins is inefficient so that, on a quantitative basis, far more Bax (ΔTM) protein production would be required to observe a functional effect on cell viability in yeast cells than in some types of mammalian cells.

In yeast and 293 cells, the wild-type Bax protein appeared to be associated largely with mitochondria. We have also observed by two-color immunofluorescence and by laser-scanning microscopy that most of the Bax protein appears to be localized to mitochondria in other human and mouse cells (unpublished data). By analogy to other Bcl-2 family proteins (14, 24, 34, 36, 48), however, association with other intracellular membranes is probably likely as well but may be underappreciated because mitochondrial association would tend to concentrate the immunofluorescence label into discrete foci and thus produce more intense signals. At issue, therefore, is the question of whether mitochondrial association is essential for Bax function, as opposed to the possibility that integration into other intracellular membranes suffices. It will be of interest in future studies therefore to purposely target Bax to other nonmitochondrial membrane locations by fusion with appropriate targeting sequences in an effort to determine whether mitochondrial association is essential.

In this regard, an important role for mitochondria in apoptosis has come from experiments involving a cell-free apoptosis system wherein mitochondria were shown to be essential for induction of apoptosis-like destruction of nuclei by cytosolic extracts (51). In that study, nuclei derived from Bcl-2-overexpressing cells were not protected from cytosolic extract-induced destruction, suggesting that Bcl-2 protein associated with the nuclear envelope was insufficient for preventing genomic digestion and nuclear destruction. Moreover, Zamzami et al. have recently reported that mitochondria isolated from preapoptotic mammalian cells release proteinaceous factors that can result in apoptotic-like destruction of naive isolated nuclei in a cell-free system in vitro (87), and it has been shown recently that cytochrome *c* may be one such mitochondrial protein (38a). Again, nuclei isolated from Bcl-2-overexpressing cells were not protected, despite evidence that nearly half of the intracellular Bcl-2 protein is associated with the nuclear envelope (36). The release of apoptotic factors from mitochondria has been linked to the induction of permeability transition (megapore opening) in mitochondria, which can be prevented by overexpression of Bcl-2 (87). Given that yeast mitochondria are also known to contain a similar channel (reviewed in reference 90), it is tempting to speculate the Bax may induce or facilitate megapore opening in both mammalian and yeast mitochondria, thereby leading to cell death. The recently determined structure of the Bcl-X_L protein, with its similarity to pore-forming bacterial toxins, is also consistent with this hypothesis (50). Perhaps analogous to the release of proteins from the mitochondria of preapoptotic cells, the pore-forming B subunit of diphtheria toxin is believed to provide a conduit for the entry of the ADP-ribosylating A subunit across lysosomal membranes into the cytosol (49). Consistent with this idea, the induction of permeability transition in mitochondria has been shown to result in release of matrix proteins (25). Of further interest is the correlation between the location of the Bcl-2 protein as determined by EM, which is enriched at the junctional complexes of mitochondria (11, 36), and data which suggest that the megapore is also associated with the junctional complexes where the inner and outer membranes of mitochondria come into contact (3). Experiments are in progress to explore the relation of Bax and Bcl-2 to regulation of mitochondrial permeability transition in yeast and mammalian cells.

Recently, Greenhalf et al. reported that Bax fails to exhibit a cytotoxic effect in petite strains of yeast ([*rho*^{0}] cells) which lack mitochondrial DNA and that therefore are incapable of accomplishing oxidative phosphorylation (17). This observation suggests a functional connection between mitochondria and the ability of Bax to confer a lethal phenotype on yeast cells. In our hands, however, Bax cytotoxicity was not impaired in [*rho*^{0}] yeast strains (unpublished data). Moreover, in the system of Greenhalf et al., Bcl-2 required a TM domain for suppression of Bax-mediated cytotoxicity, whereas in our hands the Bcl-2 (ΔTM) protein was used successfully to inhibit Bax lethality in yeast cells (this report and references 4, 19, and 61). Though strain variations could potentially account for some of these experimental differences, we have performed our analysis of Bax in three different strains (EGY48, L40, and BF264-15Dau) with similar results. In addition, previous studies have

shown that mitochondrial DNA and oxidative phosphorylation are not required for apoptosis (26). Mitochondrial DNA is also not required for establishment of a membrane potential across mitochondria or for induction of permeability transition (39). Moreover, Bcl-2 can protect [*rho*^{0}] cells from apoptosis (26). Inasmuch as suppression of apoptosis by Bcl-2 has been shown to be dependent at least in part on its ability to heterodimerize with Bax (86), it would therefore appear that oxidative phosphorylation is not essential for Bax function in mammalian cells. However, further analysis of the relevance of oxidative phosphorylation to the cell death-promoting action of Bax in yeast and mammalian cells is required before firm conclusions can be drawn.

It has been speculated that Bax-Bax homodimers promote cell death (70), but data which directly address this issue have previously been unavailable. The loss of function of the Bax (ΔIGDE) mutant seen here in both yeast and mammalian cells suggests that homodimerization may indeed be required for the cell death-promoting function of this protein. It should be recognized, however, that the removal of these four amino acids from the BH3 domain of Bax could have additional effects on other aspects of Bax protein structure and function which are unpredictable. The BH3 domain of Bax in which the IGDE motif resides has been shown recently to be required for Bax-Bax homodimerization, as well as for heterodimerization with Bcl-2 and E1b-19kDa, whereas the BH1, BH2, and N-terminal regions of Bax are expendable (18, 88). These other regions of Bax (BH1, BH2, and N) were also not essential for Bax function in yeast cells, thus lending further evidence to the argument that Bax-Bax homodimerization may be critical for its death-promoting activity in both yeast and mammalian cells. Though the Bax (ΔBH1), Bax (ΔBH2), and Bax (ΔN) mutants were not tested in mammalian cells, the proapoptotic effects of similar mutants of the Bak protein have been examined by using the same Rat-1 cell transient transfection assay system as employed here (8). Those studies demonstrated that the region of Bak where the BH3 domain resides was sufficient, in the absence of BH1, BH2, or the N domains, to promote apoptosis, though for some mutants it was necessary to include a TM domain. In addition, the proapoptotic Bcl-2 family protein Bik contains a BH3 domain but lacks entirely sequences with homology to BH1, BH2, or the N domain, further suggesting that the BH3 domain is particularly important for promotion of cell death in mammalian cells (6).

Taken together, these observations suggest at least three non-mutually exclusive models for how the BH3 domains of Bax and similar proapoptotic proteins such as Bak and Bik may regulate cell death (Fig. 9). First, this domain may be required for homodimerization of Bax and similar proapoptotic proteins, with the homodimeric forms of these proteins then somehow interacting with other currently undiscovered proteins involved in the cell death pathway or forming pores in membranes directly upon dimerization or oligomerization. Second, the BH3 domain may bind directly to some unknown cell death effector protein, thus promoting cellular demise by activating this downstream killer protein. In keeping with the non-mutually exclusive nature of these models, the BH3 domain could accomplish this in monomeric form or (as we have suggested here) may engage this killer protein only when dimerized. Third, the BH3 domain could act as a decoy that binds to Bcl-2, Bcl-X_L, or other antiapoptotic members of the Bcl-2 protein family, thus preventing them from interacting with the endogenous Bax protein, thwarting the attempts of Bcl-2 and Bcl-2-like proteins to homodimerize, or preventing Bcl-2 and its functional homologs from binding to other proteins whose interactions are critical for cell survival. To the extent that further experimentation fails to reveal homologs of Bcl-2 in yeast cells (thus far, no candidate exists), this third model could be excluded as the sole explanation for Bax protein function but probably does play a role in mammalian cells in which Bcl-2 or similar antiapoptotic proteins are expressed. As evident from the models presented in Fig. 9, Bax may employ multiple mechanisms for promoting cell death. In conclusion, the data presented here provide further evidence that the BH3 domain of Bax plays a critical role in the mechanisms by which this protein promotes cell death in both yeast and mammalian cells and also raise the possibility that homodimerization of Bax and targeting to mitochondrial membranes are important facets of its function as well.

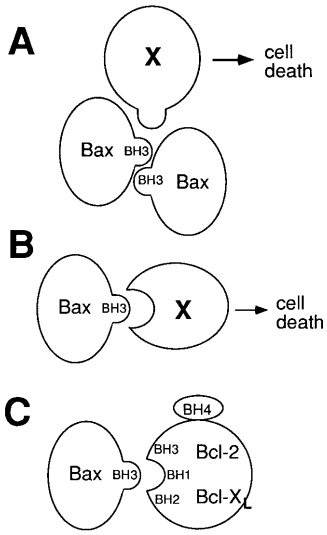


FIG. 9. Models for Bax protein function. (A) Bax homodimers (mediated by the BH3 domain) interact with an unidentified cell death effector protein (X) to promote apoptosis. (B) Bax monomer engages a cell death effector (X) via its BH3 domain (or in a BH3 domain-dependent manner). (C) The BH3 domain of Bax acts as a decoy, preventing Bcl-2 or Bcl-X_L from interacting with the endogenous Bax protein or other proteins (not shown) that are required for cell survival.

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