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Human Bak Induces Cell Death in *Schizosaccharomyces pombe* with Morphological Changes Similar to Those with Apoptosis in Mammalian Cells

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Apoptosis as a form of programmed cell death (PCD) in multicellular organisms is a well-established genetically controlled process that leads to elimination of unnecessary or damaged cells. Recently, PCD has also been described for unicellular organisms as a process for the socially advantageous regulation of cell survival. The human Bcl-2 family member Bak induces apoptosis in mammalian cells which is counteracted by the Bcl-x_L protein. We show that Bak also kills the unicellular fission yeast *Schizosaccharomyces pombe* and that this is inhibited by coexpression of human Bcl-x_L. Moreover, the same critical BH3 domain of Bak that is required for induction of apoptosis in mammalian cells is also required for inducing death in yeast. This suggests that Bak kills mammalian and yeast cells by similar mechanisms. The phenotype of the Bak-induced death in yeast involves condensation and fragmentation of the chromatin as well as dissolution of the nuclear envelope, all of which are features of mammalian apoptosis. These data suggest that the evolutionarily conserved metazoan PCD pathway is also present in unicellular yeast.

Programmed cell death (PCD) in metazoans is an essential homeostatic mechanism permitting the removal of surplus cells during morphogenesis and tissue maintenance and the deletion of cells that present a risk to the organism because they are mutated or infected (10, 19, 35, 36, 40). For vertebrates, the descriptive name commonly given to the process of PCD is apoptosis. Classical apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin condensation, and nuclear and cellular fragmentation, and it results from the activation of an intrinsic suicide program (47). Recent studies implicate the dysregulation of PCD in the pathophysiology of several human diseases, including AIDS (12, 28), neurodegenerative disease (25, 37, 44), and cancer (for a review, see reference 42).

The basal machinery responsible for metazoan PCD is highly evolutionarily conserved and, at its execution level, involves the action of a discrete class of cysteine proteases, of which the prototypes are the interleukin-1β-converting enzyme in humans and Ced-3 in the nematode (49). Also conserved are key regulators of apoptosis: in *Caenorhabditis elegans* the Ced-9 protein and in humans the Bcl-2 protein family (18, 46), which comprises both suppressors (e.g., Bcl-2 and Bcl-x_L) and promoters (e.g., Bax and Bak) of PCD (17, 30, 46).

Recently, there have been several reports describing apparent PCD in the unicellular eukaryotes *Tetrahymena thermophila*, *Dictyostelium discoideum*, *Trypanosoma brucei rhodsiense*, and *Trypanosoma cruzi* and even in bacteria (2, 6, 45, 48; for an overview, see reference 1). PCD in unicellular organisms might facilitate constant selection for the fittest cell in the colony or optimal adaptation of cell numbers to the environment or might serve as a means for altruistic cell death to prevent the spread of virus in the event of infection.

It has been shown that expression of the mammalian Bax protein in the budding yeast *Saccharomyces cerevisiae* is lethal, as defined by the inability to form colonies (13, 14, 39). This lethality is antagonized by the proteins Bcl-2, Bcl-x_L, and Mcl-1 (39), all of which suppress apoptosis in mammalian cells (17). However, the mechanism of Bax lethality in *S. cerevisiae* is unclear. Bak (for Bcl-2-homologous antagonist/killer) is another Bcl-2 family member which, like Bax, induces apoptosis in mammalian cells (5, 11, 22). Bak-induced apoptosis is suppressed by coexpression of the antiapoptotic protein Bcl-x_L (4). In this study we have examined the consequences of Bak expression in the fission yeast *Schizosaccharomyces pombe*. We show that Bak induces death of *S. pombe* cells with characteristics similar to those of mammalian apoptosis.

MATERIALS AND METHODS

Yeast culture. Yeast transformation and yeast growth experiments were performed with exponentially growing cultures. All *S. pombe* strains used were derived from the wild-type strains 972h− and 975h+. Media and growth conditions were as described previously (29).

Cloning of yeast expression constructs. A 1.2-kb cDNA fragment containing the human *bak* coding sequence was inserted into the *Bam*HI site of pREP41. Constructs containing both the sense and antisense orientations of the insert were isolated and used to transform wild-type *S. pombe*. Transformants were isolated on selective medium.

To isolate a yeast strain with stably integrated human *bak*, a 1.2-kb fragment containing the *bak* cDNA was inserted into the *Bam*HI site of pKIP45. A plasmid that contained *bak* in the sense orientation was then transformed into wild-type *S. pombe*, and transformants that grew on selective medium as large, white colonies were examined further.

A 0.7-kb fragment containing the human Bcl-x_L-coding region was inserted into the *Sma*I site of pMBS36URA Rep4 and transfected into the DSI strain of *S. pombe*. Transformants were isolated on selective medium.

Construction of *bak* deletion mutants has been described previously (4). cDNA inserts comprising these mutants were excised with *Eco*RI/*Hin*dIII, blunted with Klenow fragment, and ligated into the *Sma*I site of the yeast expression vector Rep1 or Rep41, which contain the thiamine-repressible *nmt1* promoter but differ in levels of expression that they confer.

Electron microscopy. Yeast cells were fixed in 2.5% glutaraldehyde–4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 2 h and then left overnight in 2% paraformaldehyde at 4°C. Samples were embedded in 10% gelatin which was solidified on ice. Blocks containing cells were immersed in 2.3 M sucrose in phosphate-buffered saline for 4 h and then frozen in liquid nitrogen. Ultrathin cryosections were cut on an Ultracut S microtome with an FC4E cryo attachment, transferred on to Formvar-coated grids, and then stained with uranyl

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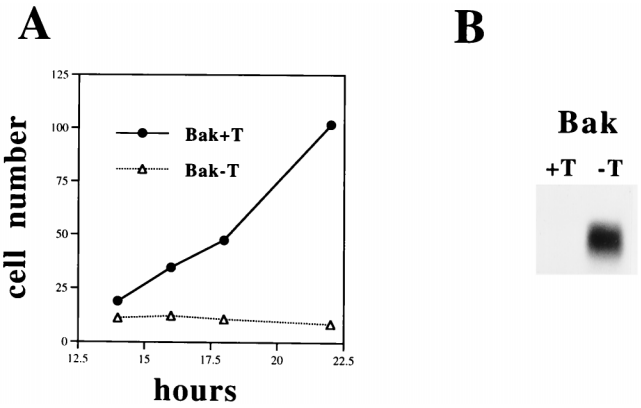

FIG. 1. Growth kinetics of *S. pombe* cells expressing Bak (DSI cells) as determined by Coulter counting. (A) DSI cells were grown in either the presence (+T) or absence (−T) of thiamine. Time zero is at the removal of thiamine, and 12 h later is the start of Bak expression. Expression of Bak inhibits growth of the culture. (B) Northern blot analysis showing induction of *bak* transcription from the *nmt-1* promoter in DSI cells upon removal of thiamine. Cells growing in the presence of thiamine do not express any *bak* RNA.

acetate and methyl cellulose as described previously (43). Sections were examined with a Jeol 1200 FX electron microscope.

Pulsed field gel electrophoresis. Cells were suspended and lysed in agarose plugs as follows. Cells were washed twice in CSE (20 mM citrate-phosphate [pH 5.6], 1.2 M sorbitol, 40 mM EDTA) and then incubated for 1 h at 37°C in 10 ml of CSE containing 1.5 mg of Zymolyase 20T (Seikagaku Kogyo) per ml. The cells were pelleted and resuspended at 6×10^8 cells per ml in TSE (10 mM Tris-HCl [pH 7.5], 0.9 M sorbitol, 45 mM EDTA). The cell suspension was warmed to 37°C, and an equal volume of 1% Incert agarose (Seakem) in TSE was added. Aliquots were dispensed into a plug mold and allowed to gel. The gelled plugs were incubated at 55°C, first for 90 min in 0.25 M EDTA–50 mM Tris-HCl (pH 7.5)–1% sodium dodecyl sulfate and then for 48 h in 1% lauryl sarcosine–0.5 M EDTA (pH 9.5)–1 mg of proteinase K per ml. The plugs were stored at 4°C in Tris-EDTA and washed three times in Tris-EDTA before loading. Pulsed-field gel electrophoresis was carried out in 0.6% chromosomal-grade agarose (Bio-Rad) in a Bio-Rad CHEF-DRII apparatus. Electrophoresis was for 48 h at 50 V in 0.5× TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA), with a switch time of 30 min.

DAPI staining. Yeast cells were fixed in ethanol, stained with DAPI (4′,6-diamido-2-phenylindole), and analyzed with a fluorescence microscope as described previously (29).

Blotting and analysis of DNA, RNA, and proteins. Southern, Northern, and Western blottings were carried out according to standard protocols (38).

RESULTS

Expression of human Bak in *S. pombe* leads to growth arrest and cell death. To express human Bak in *S. pombe*, a *bak* cDNA was subcloned downstream of the *nmt1* promoter, which is repressed when *S. pombe* is grown in minimal medium supplemented with thiamine and derepressed in thiamine-free medium (27, 29). Wild-type *S. pombe* was transformed with the *nmt1/bak* construct, and positive transformants were replica plated onto medium without thiamine containing the vital stain phloxin B. Dead yeast cells lose membrane integrity, take up phloxin B, and stain red (29). Within 24 h of plating (12 h after induction of Bak), approximately 30 to 40% of the *nmt1/bak* yeast colonies stained red; induction of Bak protein upon removal of thiamine was confirmed by immunoblotting (data not shown). In contrast, none of the colonies plated on medium with thiamine was stained, nor were any colonies transfected with an antisense *bak* construct (data not shown). Thus, Bak protein appears to exert a cytotoxic effect on *S. pombe* cells.

For further analysis, an *nmt1/bak* construct was stably integrated as a single copy in the wild-type *S. pombe* genome (single-copy integration was confirmed by Southern blotting [data not shown]). The effect of Bak expression in the stable integrant (DSI) was then examined. Upon thiamine removal, Bak protein was expressed at a relatively low level (data not shown). Nonetheless, by 16 h after thiamine removal (4 h after the onset of Bak expression), cell propagation in liquid cultures had ceased, whereas DSI cells grown in the presence of thiamine continued to grow exponentially (Fig. 1A) (Fig. 1B shows induction of *bak* mRNA in DSI cells upon thiamine removal).

Uptake of the vital dye phloxin B had suggested that Bak-expressing yeast cells (either yeast containing multiple copies of the *nmt1/bak* construct or DSI yeast cells with stably integrated *nmt1/bak*) not only cease proliferating but actually die. To confirm this notion, DSI cells grown in liquid culture, either with or without thiamine, were plated at different time points on agar containing thiamine so that Bak expression was again repressed. A high number of cells could be consistently recovered from the control culture growing in medium with thiamine. In contrast, by 10 h after the onset of Bak expression, no cells that would grow as colonies on thiamine-containing agar plates could be recovered (Fig. 2). We conclude that Bak expression in *S. pombe* causes irreversible growth arrest and cell death.

Bak-induced cell death is inhibited by Bcl-x_L. Bcl-x_L is a Bcl-2 family member which, like Bcl-2, functions to suppress apoptosis in mammalian cells. It can form heterodimers with the cell death-promoting Bcl-2 family members Bax and Bak, suggesting that this interaction may be important for the anti-apoptotic role of Bcl-x_L (4, 14). To explore the effect of Bcl-x_L on Bak-mediated cytotoxicity in yeast, a human *bcl-x_L* cDNA was placed under control of the *nmt1* promoter and transfected into strain DSI as a multicopy plasmid (to give strain DSIA). DSIA cells were then grown in minimal medium with or without thiamine. In the absence of thiamine, both Bak and Bcl-x_L were induced, and this was confirmed by Northern blotting (Fig. 3B and C). Cell numbers within the cultures were then counted at different times (Fig. 3A).

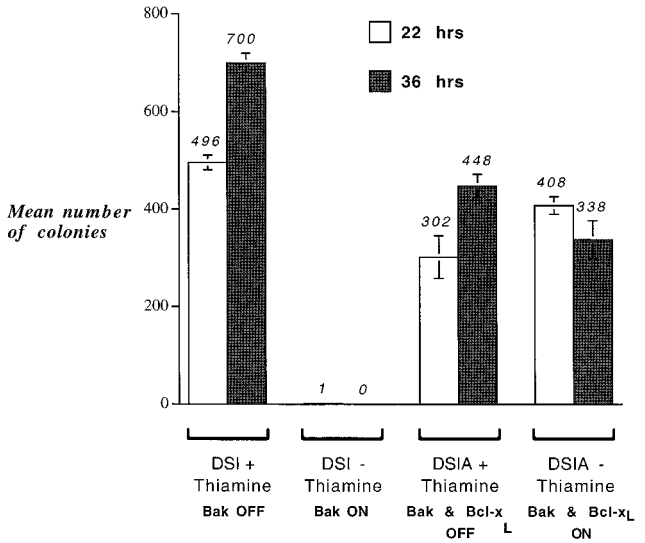

FIG. 2. Regrowth of *S. pombe* following induction of Bak or Bak and Bcl-x_L. DSI and DSIA cells were grown in liquid culture in the presence or absence of thiamine. After either 22 or 36 h (equivalent to about 10 or 24 h after Bak [DSI] or Bak plus Bcl-x_L [DSIA] induction, respectively), equal numbers of cells from each culture were plated onto agar containing thiamine, where Bak and Bcl-x_L expression were repressed again. After regrowth, colonies were counted. Each experiment was performed in duplicate. The mean numbers of colonies that grew and the standard deviations are expressed graphically, and the actual numbers are given above the bars.

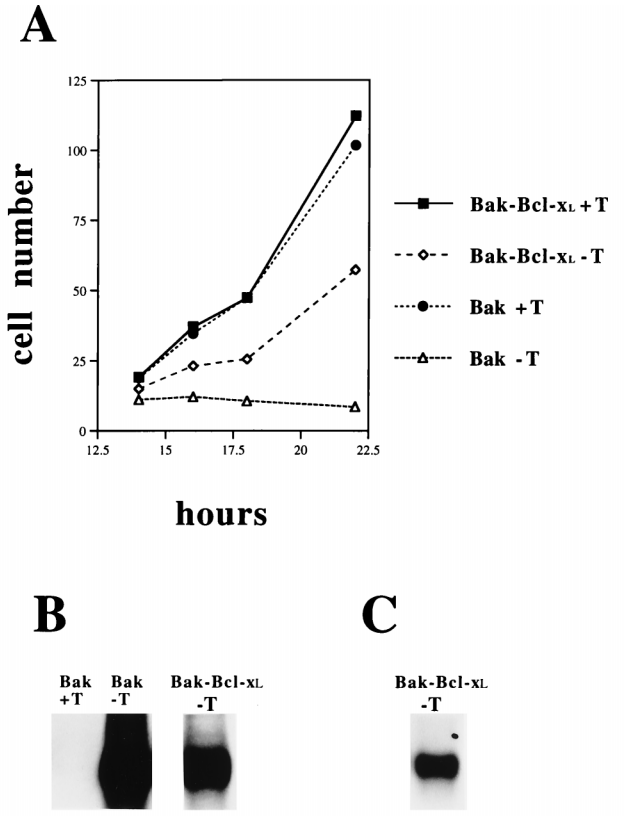

FIG. 3. Protection from Bak-induced growth arrest and cell death by coexpression of Bcl-x_L in *S. pombe*. (A) DSI (*nmt1*/*bak*) or DSIA (*nmt1*/*bak* plus *nmt1*/*bcl-x*$_L$) cells were grown in either the presence (+T) or absence (−T) of thiamine. Cell numbers in the different yeast cultures were determined at several time points by Coulter counting. The growth curves for DSI cells (Bak) are the same as shown in Fig. 1. (B) Northern blot showing induction of *bak* mRNA expression in DSI (Bak) and DSIA (Bak-Bcl-xL) cells upon removal of thiamine. (C) Northern blot analysis of *Bcl-x*$_L$ mRNA expression in DSIA cells grown in the absence of thiamine.

Compared with control cells growing in the presence of thiamine, thiamine-deprived DSIA cultures coexpressing Bak and Bcl-x_L exhibited somewhat decreased growth when examined 16 h after removal of thiamine (about 4 h after induction of Bak and Bcl-x_L). Nevertheless, growth of DSIA cultures was significantly greater than that of DSI cultures expressing Bak alone. Analysis of cell viability by vital staining with phloxin B and by assessment of colony regrowth following readdition of thiamine (Fig. 2) also showed that coexpression of Bcl-x_L effectively suppressed Bak-induced death. Importantly, expression of Bcl-x_L protein alone had no detectable effect on the growth of yeast cells (data not shown). Thus, just as in mammalian cells, Bcl-x_L mitigates the cytotoxicity of Bak in *S. pombe.*

Nuclear staining and pulsed-field gel electrophoresis reveal fragmentation and specific cleavage of chromosomal DNA in *S. pombe* cells expressing Bak. A characteristic feature of apoptosis in mammalian cells is the condensation and fragmentation of DNA (47). We therefore examined the integrity of DNA in *S. pombe* after induction of Bak. DSI cells were stained with the DNA groove-binding fluorochrome DAPI at various time points after onset of Bak expression. At 12 h after induction of *bak* transcription, the chromatin of many cells exhibited distinct fragmentation (Fig. 4). Classical mammalian apoptosis typically involves cleavage of nuclear DNA, first into

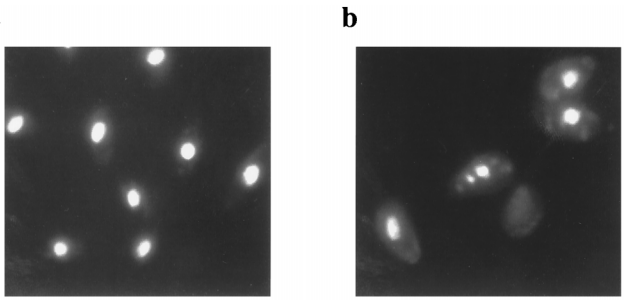

FIG. 4. Fragmentation of chromosomal DNA in *S. pombe* cells expressing Bak as shown by DAPI staining. DSI cells were stained with DAPI at 24 h after the removal of thiamine and examined by fluorescence microscopy. (a) Control DSI cells grown in the presence of thiamine; (b) DSI cells grown in the absence of thiamine.

large, 50- to 300-kb fragments (3, 34) and eventually into oligonucleosomal fragments through the action of undefined nucleases (47). DNA derived from Bak-expressing cells showed no evidence of a classical oligonucleosome ladder when fractionated on an agarose gel (data not shown). However, pulsed-field gel electrophoresis of DNA from Bak-expressing DSI cells showed the appearance of a novel high-molecular-weight DNA species (about 800 kb in size), which was not seen in control cells without Bak expression (Fig. 5). The distinct size of this DNA fragment suggests that a specific chromatin cleavage may occur during Bak-induced death in *S. pombe.*

Electron microscopy shows chromatin condensation and disappearance of the nuclear envelope during Bak-induced cell death in *S. pombe.* We next examined *S. pombe* yeast cells expressing Bak by electron microscopy. At 24 h after onset of Bak expression cells, some 10% of the DSI cells showed clear signs of nuclear condensation (Fig. 6b to e). In many cells, the nuclear membrane either appeared to be incomplete or had disappeared completely (Fig. 6e and f). Fragmentation of the nucleus, as previously observed by DAPI staining (see above), was also evident (Fig. 6b and e). The condensation of chromatin and destruction of nuclear membranes during Bak-induced death of *S. pombe* cells are strikingly similar to events occurring during apoptosis of mammalian cells. Bak-induced death of yeast cells was also accompanied by extensive vacuolization (Fig. 6f) and, in some cells, unusual mitochondrial morphology (not shown).

Analysis of Bak deletion mutants. Recently, a conserved domain in Bak (BH3, located N terminal of BH1 and BH2) that is both necessary and sufficient for cytotoxic activity in

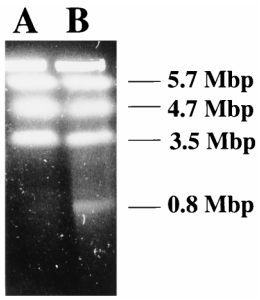

FIG. 5. Pulsed-field gel electrophoresis of genomic DNA from DSI cells incubated for 40 h in medium with (lane A) or without (lane B) thiamine. The three *S. pombe* chromosomes have sizes of 5.7, 4.7, and 3.5 Mbp, respectively. In yeast cells that express human Bak (lane B), the appearance of a 0.8-Mbp DNA fragment indicates specific DNA cleavage.

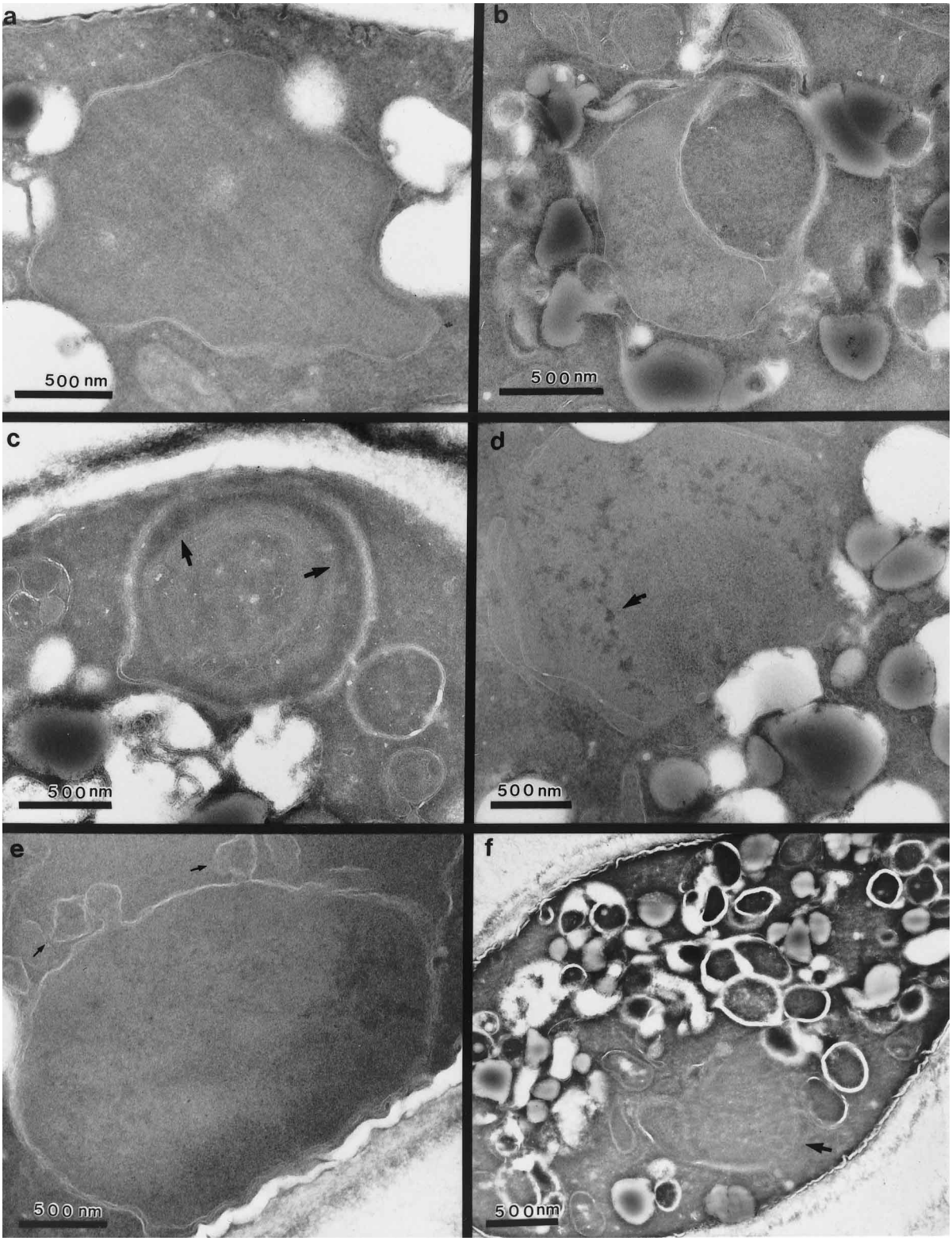

FIG. 6. Electron microscopy analysis of *S. pombe* DSI yeast cells 24 h after induction of human Bak expression. Cultures were grown in medium with thiamine (a) (no Bak expression) or without thiamine (b to f) (Bak expression). (a) Control cell with normal nuclear phenotype. (b to e) Yeast cells showing nuclear condensation (arrows in panels c and d). Panels b and e show examples of nuclear fragmentation (that in panel e implies a budding-off process of material from the nucleus [arrows]). Panel f shows a DSI cell showing heavy vacuolization and dissolution of the nuclear membrane (arrow).

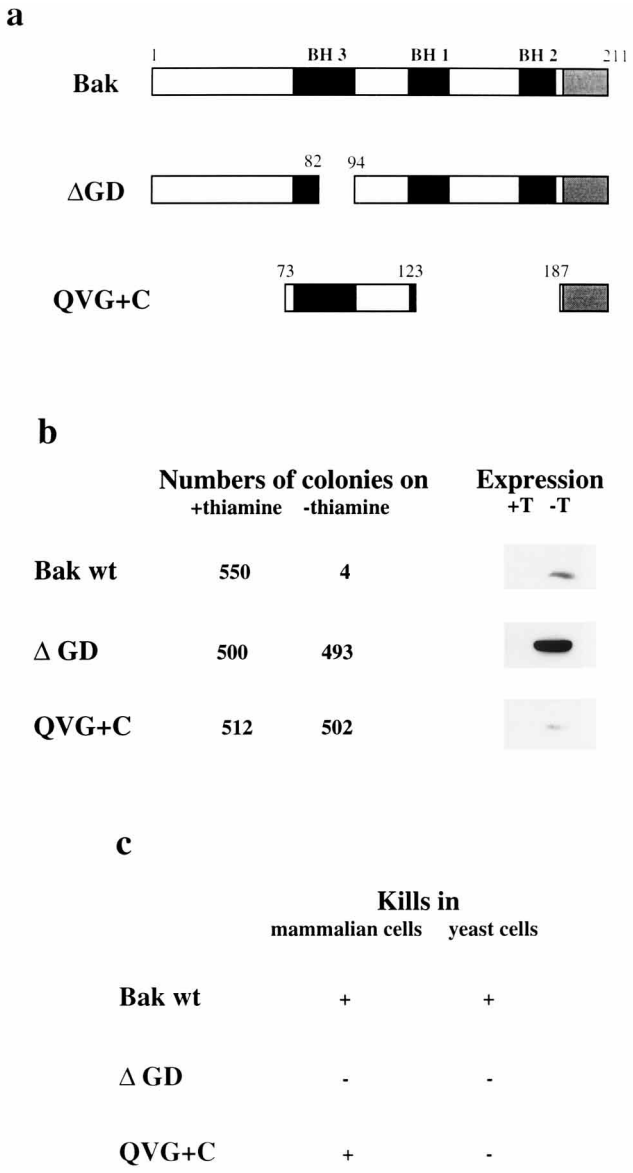

FIG. 7. Analysis of Bak deletion mutants. (a) Structures of the Bak mutants. ΔGD lacks amino acids 83 to 93, while QVG+C consists of amino acids 73 to 123 fused to the C-terminal hydrophobic end (amino acids 187 to 211). (b) Colony numbers after transfection of wild-type (wt) *bak* or deletion mutant Δ*GD* or *QVG+C* into *S. pombe* and plating on medium either with (+T) or without (−T) thiamine. Induced protein expression of wt Bak or the Bak deletion mutants upon thiamine removal was confirmed by Western blotting. wt Bak and ΔGD were detected with a polyclonal rabbit anti-Bak antibody, while for the hemagglutinin-tagged QVG+C mutant, the monoclonal antihemagglutinin antibody 12CA5 was used. (c) Summary of killing properties of wt Bak and the deletion mutants ΔGD and QVG+C. While the BH3 killing domain of Bak is necessary and sufficient to induce apoptosis in mammalian cells, it is also necessary but not sufficient to kill *S. pombe* yeast cells.

mammalian cells and for binding to Bcl-x_L was identified (4). We have used the same Bak deletion mutants to test their cytotoxic behavior in *S. pombe* cells. The various Bak mutants were placed under control of the *nmt1* promoter and transfected in *S. pombe*, and their expression was confirmed by Western blotting (Fig. 7b). The killing activity of each mutant in *S. pombe* (compared to that of wild-type Bak) was analyzed by staining with the vital stain phloxin B and by counting colonies obtained after electroporation of yeast cells with plasmids and immediate plating on minimal agar plates without thiamine.

Figure 7 depicts the structures of the deletion mutants and shows the results from a typical colony-counting experiment to determine cytotoxic efficiency. Deletion of amino acid residues 83 to 93, which encompass the BH3 killing domain required for inducing apoptosis in mammalian cells, resulted in a Bak deletion protein (ΔGD) that was no longer capable of inducing death in yeast. However, expression of the QVG+C Bak mutant, which comprises the BH3 killing domain alone fused to a hydrophobic membrane anchor (Bak amino acids 73 to 123 and 187 to 211) and is capable of both binding Bcl-x_L and inducing apoptosis in mammalian cells, proved insufficient to induce apoptosis in yeast cells. Thus, although the BH3 domain is necessary for induction of cell death in *S. pombe*, it is not sufficient for the killing activity of Bak.

DISCUSSION

An increasing number of proteins having sequence and functional homology with Bcl-2 have been identified (for a review, see reference 30), and they include proteins that either inhibit or accelerate apoptosis (30, 46). The Bcl-2 homolog Bak opposes Bcl-2 function and triggers apoptosis under certain conditions (5, 11, 22). Both Bcl-2 and Bcl-x_L bind to Bak, and this interaction is presumed to be important for suppression of Bak-induced apoptosis (4).

We have shown that Bak expression in *S. pombe* leads to growth arrest and cell death, an effect which is inhibited by coexpression of Bcl-x_L. Bak expression leads to a phenotype with typical features of mammalian apoptosis: vacuolization of the cytoplasm, chromatin condensation and fragmentation, disappearance of the nuclear envelope, and specific DNA cleavage into high-molecular-weight fragments. Overexpression of a *bak* antisense construct or of a Bak deletion mutant which lacks only the small conserved BH3 region between amino acids 83 and 93 does not result in cell death, confirming the specificity of the cytotoxic effect of Bak in *S. pombe*. BH3 alone appears to be sufficient for inducing apoptosis in mammalian cells. It is also the domain of Bak which interacts with Bcl-x_L, although it is unclear whether this provides the mechanism by which Bak kills mammalian cells. More detailed analysis of other Bak deletion mutants indicates that the BH3 domain is necessary but not sufficient for inducing cell death in *S. pombe*. We believe that our data are consistent with the presence of a conserved Bak-sensitive cell death pathway in *S. pombe*, although the regulators and constituents of such a pathway have still to be identified.

PCD not only is well characterized in metazoans but also has been identified in bacteria (31, 41, 48) and in four unicellular organisms: the kinetoplastid parasites *Trypanosoma cruzi* (2) and *Trypanosoma brucei rhodesiense* (45), the free-living slime mold *D. discoideum* (7), and the free-living ciliate *Tetrahymena thermophila* (6). PCD in these unicellular eukaryotes appears to be very similar to apoptosis in cells from multicellular organisms and features cytoplasmic blebbing and vacuolization, chromatin condensation, and DNA fragmentation. As in metazoans, survival of unicellular eukaryotes requires extracellular signals to restrain spontaneous self-destruction (1). PCD in unicellular organisms probably facilitates constant selection for the fittest cells in the colony and allows optimal adaptation of cell numbers to the environment. Given that PCD appears to exist in bacteria and in unicellular and multicellular eukaryotes, its existence in yeast may be not be too surprising.

It has been shown that overexpression of human Bax protein in the yeast *S. cerevisiae* induces growth arrest and mortality

(13, 14, 39). This toxicity can be overcome by coexpressing Bcl-2 or Bcl-x_L. Some evidence indicates that the rescuing effect of these antiapoptotic proteins is linked to yeast mitochondrial function (13). The phenotype of Bax killing in *S. cerevisiae* has recently been further described (50), and it is well possible that the mechanism involved in this lethality is the same as that in *S. pombe* cells killed by Bak expression. This might further strengthen the possibility of a conserved yeast PCD pathway.

Recently, a signal transduction pathway that uses the lipid ceramide as a second messenger has been identified in the yeast *S. cerevisiae* (32). In mammalian cells several growth modulators, including tumor necrosis factor alpha, interleukin-1β, gamma interferon, and vitamin D_3, transduce their biological activities at least in part through the ceramide pathway (15, 23, 24; for a review, see reference 16). Agonist-induced hydrolysis of plasma membrane sphingomyelin to ceramide by a sphingomyelinase results in ceramide production and accumulation. The immediate targets of ceramide signalling are a ceramide-activated protein kinase (26) and a ceramide-activated protein phosphatase (9). The various biological responses of different target cells to activation of the ceramide signalling cascade (depending on cell type) are all of a generally antiproliferative nature and include Rb-mediated cell cycle arrest, induction of apoptosis, and initiation of terminal differentiation (8, 16, 20, 21, 33). An antiproliferative G_1 arrest response has also been observed in the yeast *S. cerevisiae* upon ceramide treatment (32). Moreover, *S. cerevisiae* cells contain a ceramide-activated protein phosphatase (equivalent to the mammalian ceramide-activated protein phosphatase) whose function appears to be necessary for the observed ceramide growth inhibition. It will be interesting to determine whether this conservation of the ceramide-mediated antiproliferative signal transduction pathway between yeast and mammalian cells includes activation of a conserved yeast PCD program.

In regard to apoptosis, one of the most pressing unanswered questions concerns the function of the Bcl-2 family proteins. Bcl-2 and Bcl-x_L both protect cells and form heterodimers with Bak and Bax, both of which kill. Unfortunately, we do not know whether Bcl-2 and Bcl-x_L block a killing function of Bax and Bak or whether Bak and Bax suppress a protective action of Bcl-2 and Bcl-x_L. In this study, we have shown that Bak kills *S. pombe*, an observation most consistent with the notion that Bak is a promoter of cell death whose action is mitigated by Bcl-x_L.

Bak killing of yeast could occur either by a mechanism homologous to the way it induces apoptosis in mammalian cells or merely because Bak is a foreign protein that interferes nonspecifically with yeast physiology. We favor the former model for the following reasons. First, Bak lethality requires the same critical BH3 domain as is required for killing in mammalian cells. Second, Bcl-x_L, a relative of Bak that is arguably equally foreign to yeast, not only fails to kill *S. pombe* cells but actually suppresses the lethal action of Bak, exactly as in mammalian cells. Nonetheless, analysis of Bcl-2 deletion mutants in *S. cerevisiae* indicates that not all mutants that retain ability to heterodimerize with Bax in vitro will neutralize Bax-mediated cytotoxity (14). Thus, it is possible that Bcl-2 and Bcl-x_L may exert some death-protective effect in yeast that is independent of heterodimerization with Bax and may involve direct interference with some internal yeast PCD pathway.

The effective and rapid killing activity of Bak in *S. pombe* provides the basis for a powerful yeast-based functional genetic screen of apoptosis-inhibiting proteins. We are currently analyzing a number of *S. pombe* isolates that have become resistant to Bak-induced cell death following transfection with a mouse expression cDNA library (51). Analogous screening of an *S. pombe* expression cDNA library might result in identification of proteins that regulate the viability of yeast cells.

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B.I. and M.Z. contributed equally to this work.

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REFERENCES

1. **Ameisen, J.** 1996. The origin of programmed cell death. Science **272:**1278–1279.
2. **Ameisen, J., T. Idziorek, O. Billaut-Mulot, M. Loyens, J.-P. Tissier, A. Potentier, and A. Ouaissi.** 1995. Apoptosis in a unicellular eukaryote (*Trypanosoma cruzi*): implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival. Cell Death Differ. **2:**285–300.
3. **Brown, D. G., X. M. Sun, and G. M. Cohen.** 1993. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J. Biol. Chem. **268:**3037–3039.
4. **Chittenden, T., C. Flemington, A. B. Houghton, R. G. Ebb, G. J. Gallo, B. Elangovan, G. Chinnadurai, and R. J. Lutz.** 1995. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J. **14:**5589–5596.
5. **Chittenden, T., E. Harrington, R. O'Connor, G. Evan, and B. Guild.** 1995. Induction of apoptosis by the Bcl-2 homologue Bak. Nature **374:**733–736.
6. **Christensen, S., D. Wheatley, M. Rasmussen, and L. Rasmussen.** 1995. Mechanism controlling death, survival and proliferation in a model unicellular eukaryote *Tetrahymena thermophila*. Cell Death Differ. **2:**301–308.
7. **Cornillon, S., C. Foa, J. Davoust, N. Buonavista, J. D. Gross, and P. Golstein.** 1994. Programmed cell death in Dictyostelium. J. Cell Sci. **107:**2691–2704.
8. **Dbaibo, G. S., M. Y. Pushkareva, S. Jayadev, J. K. Schwarz, J. M. Horowitz, L. M. Obeid, and Y. A. Hannun.** 1995. Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. Proc. Natl. Acad. Sci. USA **92:**1347–1351.
9. **Dobrowsky, R. T., and Y. A. Hannun.** 1992. Ceramide stimulates a cytosolic protein phosphatase. J. Biol. Chem. **267:**5048–5051.
10. **Ellis, R. E., J. Y. Yuan, and H. R. Horvitz.** 1991. Mechanisms and functions of cell death. Annu. Rev. Cell Biol. **7:**663–698.
11. **Farrow, S., J. White, I. Martinou, T. Raven, K.-T. Pun, C. Grinham, J.-C. Martinou, and R. Brown.** 1995. Cloning of a novel *bcl*-2 homologue by interaction with adnovirus E1B 19K. Nature **374:**731–733.
12. **Gougeon, M. L., and L. Montagnier.** 1993. Apoptosis in AIDS. Science **260:**1269–1270. (Erratum, **260:**1709.)
13. **Greenhalf, W., C. Stephan, and B. Chaudhuri.** 1996. Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in Saccharomyces cerevisiae. FEBS Lett. **380:**169–175.
14. **Hanada, M., C. Aime Sempe, T. Sato, and J. C. Reed.** 1995. Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. J. Biol. Chem. **270:**11962–11969.
15. **Hannun, Y. A.** 1994. The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. **269:**3125–3128.
16. **Hannun, Y. A., and R. M. Bell.** 1993. The sphingomyelin cycle: a prototypic sphingolipid signaling pathway. Adv. Lipid Res. **25:**27–41.
17. **Harrington, E., A. Fanidi, and G. Evan.** 1994. Oncogenes and cell death. Curr. Opin. Genet. Dev. **4:**120–129.
18. **Hengartner, M., and H. Horvitz.** 1994. C. *elegans* cell survival gene *ced*-9 encodes a functional homolog of the mammalian proto-oncogene *bcl*-2. Cell **76:**665–676.
19. **Hurle, J. M.** 1988. Cell death in developing systems. Methods Achiev. Exp. Pathol. **13:**55–86.
20. **Jarvis, W. D., R. N. Kolesnick, F. A. Fornari, R. S. Traylor, D. A. Gewirtz, and S. Grant.** 1994. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc. Natl. Acad. Sci. USA **91:**73–77.
21. **Jayadev, S., B. Liu, A. E. Bielawska, J. Y. Lee, F. Nazaire, M. Pushkareva, L. M. Obeid, and Y. A. Hannun.** 1995. Role for ceramide in cell cycle arrest. J. Biol. Chem. **270:**2047–2052.
22. **Kiefer, M., M. J. Brauer, V. C. Powers, J. J. Wu, S. R. Umansky, L. D. Tomei, and P. J. Barr.** 1995. Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature **374:**736–739.
23. **Kolesnick, R.** 1992. Ceramide: a novel second messenger. Trends Cell Biol. **2:**233–236.
24. **Kolesnick, R., and D. W. Golde.** 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell **77:**325–328.
25. **Liston, P., N. Roy, K. Tamai, C. Lefebvre, S. Baird, G. Cherton Horvat, R.**

Farahani, M. McLean, J. E. Ikeda, A. MacKenzie, and R. G. Korneluk. 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature **379:**349–353.
26. **Liu, J., S. Mathias, Z. Yang, and R. N. Kolesnick.** 1994. Renaturation and tumor necrosis factor-alpha stimulation of a 97-kDa ceramide-activated protein kinase. J. Biol. Chem. **269:**3047–3052.
27. **Maundrell, K.** 1990. nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J. Biol. Chem. **265:**10857–10864.
28. **Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijnster, R. P. Keet, and F. Miedema.** 1992. Programmed death of T cells in HIV-1 infection. Science **257:**217–219.
29. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. **194:**795–823.
30. **Möröy, T., and M. Zörnig.** 1997. Regulators of life and death: the *bcl*-2 gene family. J. Cell Phys. Biochem. **6:**312–336.
31. **Naito, T., K. Kusano, and I. Kobayashi.** 1995. Selfish behavior of restriction-modification systems. Science **267:**897–899.
32. **Nickels, J. T., and J. R. Broach.** 1996. A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of Saccharomyces cerevisiae. Genes Dev. **10:**382–394.
33. **Obeid, L. M., C. M. Linardic, L. A. Karolak, and Y. A. Hannun.** 1993. Programmed cell death induced by ceramide. Science **259:**1769–1771.
34. **Oberhammer, F., J. Wilson, C. Dive, I. Morris, J. Hickman, A. Wakeling, P. Walker, and M. Sikorska.** 1993. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosoma fragmentation. EMBO J. **12:**3679–3684.
35. **Oppenheim, R. W.** 1991. Cell death during development of the nervous system. Annu. Rev. Neurosci. **14:**453–501.
36. **Raff, M. C.** 1992. Social controls on cell survival and cell death. Nature **356:**397–400.
37. **Roy, N., M. S. Mahadevan, M. McLean, G. Shutler, Z. Yaraghi, R. Farahani, S. Baird, A. Besner Johnston, C. Lefebvre, X. Kang, et al.** 1995. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell **80:**167–178.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sato, T., M. Hanada, S. Bodrug, S. Irie, N. Iwama, L. H. Boise, C. B. Thompson, E. Golemis, L. Fong, H. G. Wang, et al.** 1994. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc. Natl. Acad. Sci. USA **91:**9238–9242. (Erratum, **92:**2016, 1995.)
40. **Saunders, J., Jr., and J. Fallon.** 1967. Cell death in morphogenesis, p. 289–314. *In* M. Locke (ed.), Major problems in developmental biology. Academic Press, New York, N.Y.
41. **Shub, D. A.** 1994. Bacterial viruses. Bacterial altruism? Curr. Biol. **4:**555–556.
42. **Sluyser, M.** 1996. Apoptosis in normal development and cancer. Taylor & Francis, London, United Kingdom.
43. **Tokuyasu, T.** 1989. Use of poly (vinylpyrrolidone) and poly (vinyl alcohol) for cryo ultramicrotomy. Histochem J. **21:**163–171.
44. **Vito, P., E. Lacana, and L. D'Adamio.** 1996. Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. Science **271:**521–525.
45. **Welburn, S., C. Dale, D. Ellis, R. Beecroft, and T. Pearson.** 1996. Apoptosis in procyclic *Trypanosoma brucei rhodsiense* in vitro. Cell Death Differ. **3:**229–236.
46. **White, E.** 1996. Life, death, and the pursuit of apoptosis. Genes Dev. **10:**1–15.
47. **Wyllie, A.** 1992. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metas. Rev. **11:**95–103.
48. **Yarmolinsky, M. B.** 1995. Programmed cell death in bacterial populations. Science **267:**836–837.
49. **Yuan, J. Y., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The *C. elegans* cell-death gene *ced*-3 encodes a protein similar to mammalian interleukin-1 beta converting enzyme. Cell **75:**641–652.
50. **Zha, H. B., H. A. Fisk, M. P. Yaffe, N. Mahajan, B. Herman, and J. C. Reed.** 1996. Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol. Cell. Biol. **16:**6494–6508.
51. **Zörnig, M., and G. Evan.** Unpublished data.