Mammalian Bax triggers apoptotic changes in yeast

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Abstract Apoptosis is co-regulated by the conserved family of Bcl-2-related proteins, which includes both its agonists (Bax) and antagonists (Bcl-X_L). A mutant strain of the yeast *Saccharomyces cerevisiae* has been shown to express all morphological signs of apoptosis. Overexpression of Bax is lethal in *S. cerevisiae*, whereas simultaneous overexpression of Bcl-X_L rescues the cells. We report that overexpression of mammalian Bax in a *S. cerevisiae* wild type strain triggers morphological changes similar to those of apoptotic metazoan cells: the loss of asymmetric distribution of plasma membrane phosphatidylserine, plasma membrane blebbing, chromatin condensation and margination, and DNA fragmentation. Simultaneous overexpression of Bcl-X_L prevents these changes. We demonstrate that Bax triggers phenotypic alterations in yeast strongly resembling those it causes in metazoan apoptotic cells.

Key words: Apoptosis; Bax; Yeast *Saccharomyces cerevisiae*

1. Introduction

Apoptosis, a form of programmed cell death, is a part of normal development and homeostasis in metazoans. Breakdown of its regulation leads to a variety of diseases such as cancer, and neurodegenerative and autoimmunity disorders [1]. Morphologically apoptosis is defined by the loss of asymmetric distribution of plasma membrane phospholipids [2], condensation and margination of chromatin, DNA fragmentation [3], plasma membrane blebbing, and finally the breakage of the cell into membrane-enclosed apoptotic bodies [4]. This process is co-regulated by the conserved family of Bcl-2-related proteins, which includes both agonists (Bax, Bak, Bad, Bcl-X_S) and antagonists (Bcl-2, Bcl-X_L) of apoptosis [5]. The decision on entry to apoptosis is achieved by direct interaction between these two groups. This interplay may regulate the constitution of channels in intracellular membranes and interaction with additional proteins [6]. The ultimate purpose of these interactions in mammalian cells is to control the activation of caspases. These are a group of cysteine proteases thought to be the actual effectors of apoptosis [7], which cleave specific target substrates [8].

A model has emerged recently, according to which the pro-apoptotic members of the Bcl-2 family trigger cell death by facilitating the release of cytochrome *c* from mitochondria. Cytochrome *c* then binds to CED-4/Apaf-1, an adaptor protein which in turn processes caspase-9/CED-3 to its active form. Negative apoptotic regulators of the Bcl-2 family are suggested to act at two levels. They are able to block release of cytochrome *c*, or prevent the activation of the caspase cascade by binding to CED-4/Apaf-1 [9].

While studying the interaction of Bax with other Bcl-2-like proteins, Reed and colleagues observed that its overexpression inhibited the growth of the yeast *Saccharomyces cerevisiae*, a phenotype which could be suppressed by simultaneous overexpression of antiapoptotic members of the Bcl-2 family [10,11]. This no-growth phenotype was demonstrated to be caused by cell death, and not simply by growth arrest [12]. These observations led to the key question: is the death triggered in yeast by metazoan apoptotic regulators equivalent to apoptosis? The ensuing studies gave equivocal results [13–15]. Recently a mutation in an indigenous *S. cerevisiae* protein Cdc48p has been described, which caused a set of phenotypic changes in yeast normally defining apoptosis in metazoan cells [16]. We therefore asked if overexpression of the mammalian protein Bax leads to a similar apoptotic phenotype in this unicellular eukaryote.

2. Materials and methods

2.1. Yeast strains and plasmids

Plasmids pSD10.a-Bax and pSD10.a-Bcl-X_L [17] contain a cDNA fragment of the respective gene under the control of a hybrid GAL1-10/CYC1 promoter in a pRS316-based vector with URA3 marker [18]. The marker in pSD10.a-Bcl-X_L was changed to LEU2, yielding pL009. Wild type *S. cerevisiae* strain WCG4 (*MATα his3-11,15 leu2-3,112 ura3*) was transformed with plasmids pSD10.a-Bax (CEN6, URA3), pL009 (CEN6, LEU2), and control vectors pRS315 (CEN6, LEU2) and pRS316 (CEN6, URA3) [19]. Strains were pregrown in synthetic complete (SC) medium (0.67% nitrogen base without amino acids, amino acids and nucleotide bases) lacking leucine and uracil, and containing 2% glucose as the carbon source at 30°C to a cell density of about 0.5×10^6/cm^3. To induce the expression of Bax and Bcl-X_L, cells were washed three times and resuspended in SC medium with 2% galactose instead of glucose. Cells with control plasmids and with Bax plus Bcl-X_L plasmids were diluted so that the cell density at the end of treatment was about 0.5×10^6/cm^3. The cell density of the culture of cells expressing Bax did not change within 22 h of further incubation.

To visualize nuclei the cells were incubated with 1 μg/cm^3 DAPI in HEPES buffer (10 mM HEPES/NaOH buffer pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 20 min, washed three times with HEPES buffer, and examined under the fluorescence microscope.

2.2. Annexin V staining

Externalization of PS was detected as described previously [16]. Cells were resuspended in digestion buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM PO_4^{3-}, pH 6.8) and incubated for 2 h at 30°C with 15 U/cm^3 zymolyase 100T (Seikagaku Corporation, Tokyo, Japan) and

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Abbreviations: DAPI, diaminophenylindole; PI, propidium iodide; PS, phosphatidylserine; SC, synthetic complete growth medium; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

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5.5% glusulase (NEN, Boston, MA). After cell wall digestion cells were washed in binding buffer containing sorbitol (1.2 M sorbitol, 10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The protoplasts were resuspended in 38 μl of binding buffer and incubated with 2 μl annexin-FITC (Clontech) and 2 μl PI (500 μg/ml) in the dark for 20 min at room temperature. Cells were mounted on a slide and examined under the fluorescence microscope.

2.3. Electron microscopy

Yeast cells were prepared for electron microscopy as described previously [16]. Cells were fixed with phosphate-buffered glutardialdehyde, cell walls were removed, and the cells were postfixed with osmium tetroxide and uranyl acetate and dehydrated as described by Byers and Goetsch [20] for stationary-phase cells. Following the 100% ethanol washes, cells were washed with 100% acetone, infiltrated with 50% acetone/50% Epon for 30 min and with 100% Epon for 20 h. Cells were transferred to fresh 100% Epon and incubated at 56°C for 48 h before cutting thin sections and staining with lead acetate.

2.4. TUNEL staining

A previously described protocol for TUNEL detection of fragmented nuclear DNA in yeast [16] was used with small changes. Cells were fixed in 3.7% formaldehyde for 1 h and the cell walls were removed as described above. The protoplasts were then applied to polylysine-coated slides. In Situ Cell Death Detection Kit POD (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions. After mounting the coverslip with a drop of Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) the cells were examined under the light microscope.

3. Results

3.1. Bax causes the loss of plasma membrane asymmetry in yeast

Lipid composition of the inner and outer leaflets is different in most biological membranes [21], including the yeast plasma membrane [22]. This asymmetry is maintained by an enzymatic activity [23,24] linked in both mammalian and yeast cells to ABC transporters [25,26]. In cells undergoing apoptosis the asymmetry of the plasma membrane is lost and phosphatidylserine (PS) is exposed on the cell surface [2]. In mammalian cells, this process is enhanced by the loss of the aminophospholipid translocase activity and the presence of extracellular calcium [27].

To test if overexpression of Bax in budding yeast leads to the loss of membrane asymmetry and the exposure of PS on the cell exterior, protoplasts were incubated with FITC-labeled annexin V, a PS binding protein. In parallel, the intactness of the protoplasts was assessed by their ability to exclude propidium iodide (PI). After 6 h of galactose induction the binding of annexin V was visible in some cells as punctate structures at the plasma membrane (Fig. 1a), after 15 h about 10% of the cells showed green halos surrounding the cells (Fig. 1c,e).

Exposure of PS on the cell surface accompanying the Bax-induced death of yeast may be due to loss of the putative aminophospholipid translocase activity in a process similar to PS exposure in apoptotic mammalian cells. In mammalian systems the display of PS on the cell surface serves as a recognition signal for removal of apoptotic cells by macrophages [28]. The presence of this phenomenon already in yeast indicates a more fundamental connection to apoptosis.

3.2. Overexpression of Bax leads to chromatin condensation and plasma membrane blebbing

Bax expression induced changes of nuclear morphology of yeast cells were followed by diaminophenylindole (DAPI) staining of DNA. After overexpression of Bax for 15 h about 20% of the cells showed abnormal nuclear morphology: alterations ranged from accumulation of DAPI staining in ring-like structures (Fig. 2e), presence of multiple DAPI-stained regions within the area of the nucleus (Fig. 2c), to the appearance of multiple stained regions within a single cell (Fig. 2a).

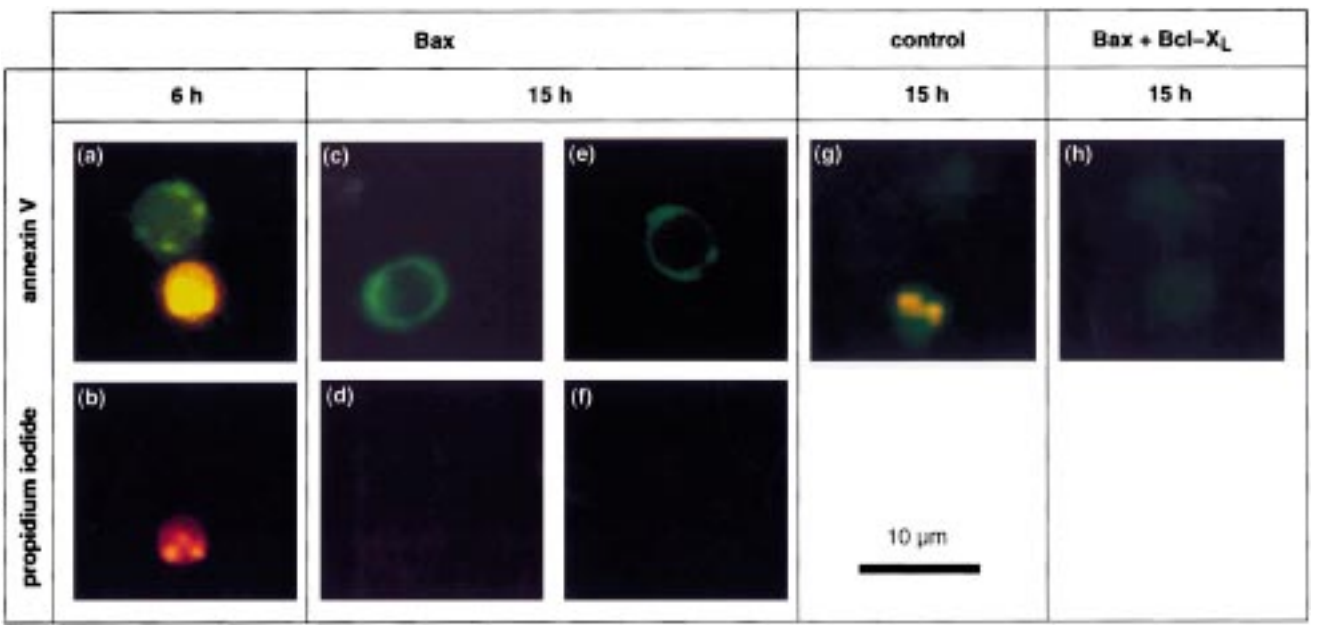

Fig. 1. Overexpression of Bax leads to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane of *S. cerevisiae* cells. Cells transformed with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a–f), control plasmids pRS315 and pRS316 (g), or plasmids pSD10.a-Bax and pL009 carrying Bcl-X_L (h). Cells were probed with FITC-annexin V conjugate and propidium iodide after 6 h (a,b) or 15 h (c–h) of induction on galactose medium. Figures (a,b), (c,d), (e,f) show the same cells examined with the FITC (a,c,e,g,h) and PI (b,d,f) filters. Cells binding annexin V exclusively on the outer leaflet of the plasma membrane are recognized by a green halo and no orange staining.

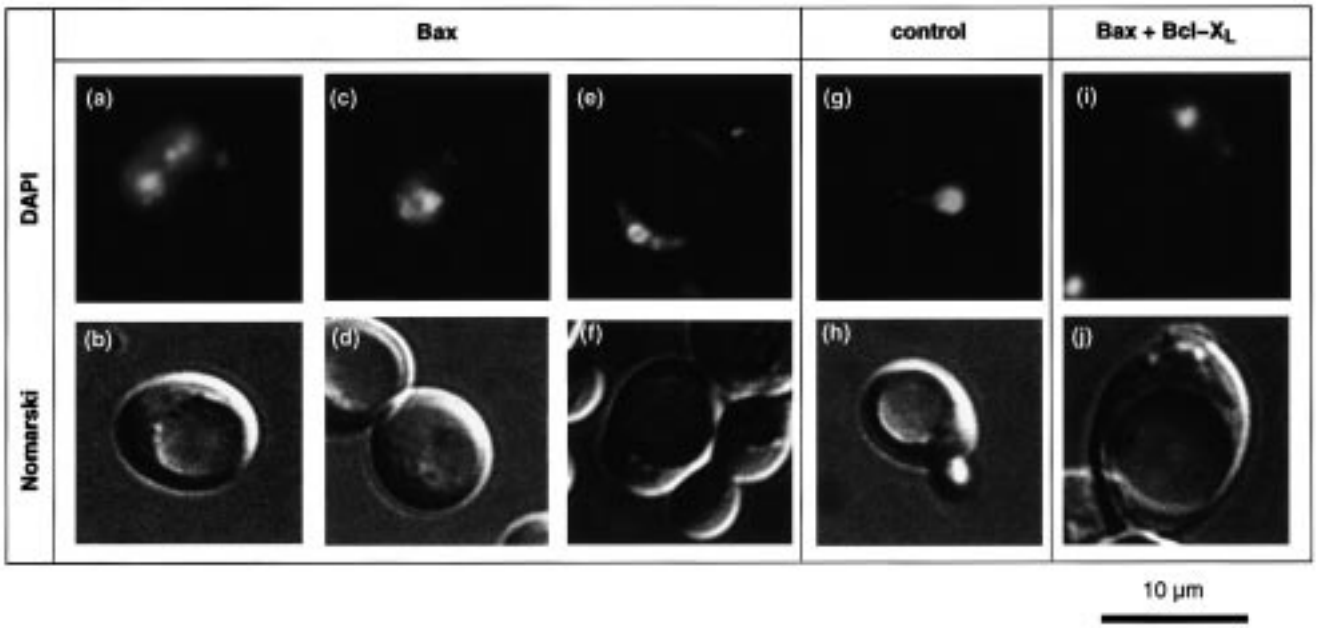

Fig. 2. Nuclei of cells overexpressing Bax show abnormal morphology. Yeast cells containing the control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a–f), control plasmids pRS315 and pRS316 (g,h), or plasmids pSD10.a-Bax and pL009 carrying Bcl-X_L (i,j) were stained with DAPI after 15 h of induction on galactose. Figures (a,b), (c,d), (e,f), (g,h), (i,j) show the same cells examined with the DAPI filter (a,c,e,g,h,i) and Nomarski optics (b,d,f,h,j).

Electron microscopic examination of same culture as used for DAPI staining revealed the presence of nuclear regions of condensed chromatin, often located at the periphery of the nucleus (Fig. 3c,d). These alterations in the nuclear morphology are reminiscent of the changes observed in apoptotic mammalian cells [29], and very similar to those described in the yeast *cdc48*^{S565G} mutant [16].

Chromatin condensation as a result of expression of Bax or its homologue Bak had been observed in *Schizosaccharomyces pombe* [14,15]. However, changes in nuclear morphology reported by these groups were accompanied by extensive vacuolization, perhaps induced by prolonged expression of pro-apoptotic proteins (for up to 48 h). This may mirror a situation during terminal stages of mammalian apoptosis when the cellular content is being disposed of. On the other hand, when ced-4 was expressed in *S. pombe* for only 12 h, extensive chromatin condensation and disruption of the nucleus took place in the absence of any vacuolization [30]. Under our experimental conditions no vacuolization was observed. Only some degree of plasma membrane blebbing occurred (Fig. 3c), paralleling the situation during the onset of mammalian apoptosis.

3.3. Nuclear DNA is fragmented in yeast overexpressing Bax

To examine if the alterations in nuclear morphology are also accompanied by changes in the physical intactness of DNA, we inspected the Bax expressing cells by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assay [31]. During this procedure terminal deoxynucleotidyl transferase attaches FITC-conjugated dUTP tails to free 3′ ends of DNA in a template-independent fashion. With the breakage of DNA the number of free 3′ ends increases significantly, giving rise to a stronger TUNEL staining in the affected nuclei.

When performing the TUNEL assay, approximately 30% of the Bax-expressing cells showed staining in their nuclei (Fig. 4a), indicating the presence of damaged DNA. A similar rate of TUNEL staining had been found in the exponentially growing *cdc48*^{S565G} mutant [16]. In mammalian cells the cleavage of DNA as a result of Bax-induced apoptosis is thought to be dependent on the activity of caspases [32] and caspase-activated DNase(s) [8]. In bacteria, on the contrary, the expression of Bax led to DNA damage in the absence of caspases [33], as in our yeast system.

3.4. Bcl-X_L prevents the appearance of Bax-induced apoptotic phenotypes in yeast

Bcl-X_L prevents Bax-induced cell death in both metazoan and yeast cells. We observed that co-expression of Bax and Bcl-X_L not only rescues the yeast cells from Bax-induced cell death, but also prevents the characteristic phenotypes that accompany it. We observed no binding of annexin V to the intact protoplasts (Fig. 1h). The morphology of the nucleus examined by DAPI staining appeared wild type-like (Fig. 2i), as well as the appearance of the nuclei under the electron microscope (Fig. 3b). In addition no significant TUNEL staining was detected in the nuclei of these cells (Fig. 4c). These data show that the phenotypes associated with Bax overexpression in yeast are tightly linked with its killing function, since co-expression of its antagonist, Bcl-X_L, eliminated both the Bax-induced cell death and its associated phenotypes.

4. Discussion

S. cerevisiae cells overexpressing the mammalian apoptotic stimulator Bax display a series of changes, identical to morphological markers defining apoptosis in mammalian cells. Early during Bax induction the plasma membrane of the cells apparently loses its asymmetry as PS is exposed on the surface of the cell. Chromatin undergoes condensation and margin-

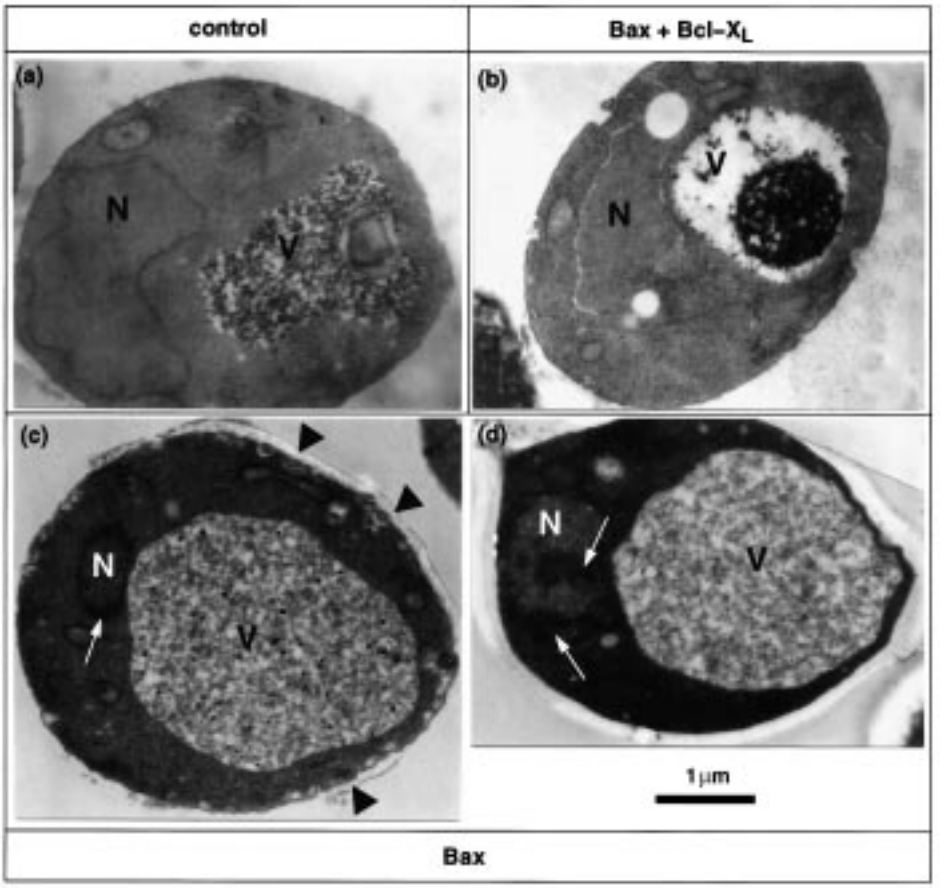

Fig. 3. Overexpression of Bax results in chromatin margination and condensation, as well as blebbing of the yeast plasma membrane. Electron micrographs of yeast cells transformed with control plasmids pRS315 and pRS316 (a), plasmids pSD10.a-Bax and pL009 carrying Bax and Bcl-X_L, respectively (b), or with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (c,d). Cells were induced on galactose for 15 h. Arrows point to regions of nuclei with condensed and marginalized chromatin, arrowheads mark the blebbing of the plasma membrane. N, nucleus; V, vacuole.

ation, and nuclear DNA is fragmented. Some degree of plasma membrane blebbing was observed as well. These alterations are absent in the control cells cultivated under identical conditions, as well as in the cells where the lethal effect of Bax expression was compensated by co-expression of the mammalian apoptotic inhibitor Bcl-X_L. In a screen for genes involved in the putative apoptotic pathway in yeast only 9% of the clones that could be induced to undergo cell death showed positive annexin V and TUNEL staining (M. Ligr and F. Madeo, unpublished observations). Therefore the observed

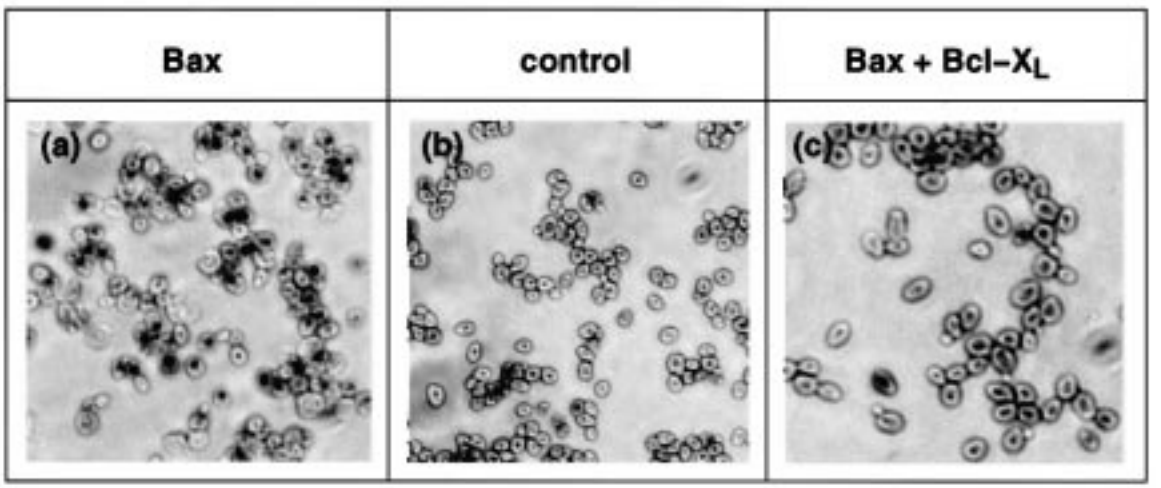

Fig. 4. The nuclei of yeast cells overexpressing Bax contain fragmented DNA. Yeast cells transformed with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a), control plasmids pRS315 and pRS316 (b), and plasmids pSD10.a-Bax and pL009 carrying Bax and Bcl-X_L (c) were induced on galactose for 15 h. Fragmentation of nuclear DNA was detected with the TUNEL test (formation of black-stained nuclei).

apoptotic phenotypes are not likely to be just general features of dying yeast. We do not see any connection among the observed phenotypes of Bax-killed yeast cells other than their link to metazoan apoptosis.

The effects of Bax expression in *S. pombe* and *S. cerevisiae* reported previously were based largely on electron microscopy and led to conclusion that Bax-induced cell death in yeast differs from apoptosis [13–15]. In contrast, using a comprehensive set of tests for morphological markers of apoptosis, we show that Bax-induced cell death in budding yeast has an apoptotic character.

The apoptotic program in metazoan cells is thought to depend on the proteolytic action of caspases [7]. The complete sequence of the yeast genome [34] confirmed the absence of homologues of known apoptotic regulators of the Bcl-2 family, as well as of caspases in *S. cerevisiae*. Interestingly, Bax is able to kill mammalian cells in the absence of caspase activity as well [32]. In this case the lethal effect of Bax is accompanied by the production of reactive oxygen species.

These findings point to the presence of a pathway responsible for execution of cell death common in yeast and metazoan cells. This putative pathway could function in parallel with the well studied caspase-dependent pathway in multicellular eukaryotes, or it could be a basic framework upon which the caspase pathway developed.

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