Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-x_L

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Received 28 July 1997; revised version received 7 August 1997

Abstract The characteristics of mitochondria of yeast cells expressing the pro-apoptotic gene Bax or coexpressing Bax and the anti-apoptotic gene Bcl-x_L have been investigated in whole cells, isolated mitochondria and permeabilized spheroplasts. It is found that Bax-induced growth arrest of yeast cells is related to two defects in the respiratory chain: (i) a decrease in the amount of cytochrome c oxidase, the terminal enzyme of the respiratory chain, and (ii) a dramatic increase in the release of cytochrome c to the cytosol. Other components of the inner mitochondrial membrane (bc_1 complex and F_0F_1-ATPase) are unaffected. Coexpression of Bcl-x_L almost fully prevented the effect of Bax. Surprisingly, these results obtained in yeast parallel similar observations reported in mammalian cells.

Key words: Apoptosis; Bax; Bcl-x_L; Cytochrome c; Mitochondrion; *Saccharomyces cerevisiae*

1. Introduction

The Bcl-2 gene product is a potent inhibitor of apoptotic cell death [1]. It is a member of the growing family of Bcl-2-like proteins, some of which inhibit apoptosis (e.g. Bcl-x_L, the long form of Bcl-x [2]), while others are involved in inducing apoptosis (e.g. Bax [3]). There exist in invertebrates proteins that are similar to members of the Bcl-2 family (e.g. ced9, the Bcl-2 homolog in *Caenorhabiditis elegans* [4]). Until now, proteins similar to Bcl-2 or Bax have not been found in lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*. Accordingly, no phenomenon resembling apoptosis has been described in yeast until now.

Yeast is a powerful tool for studying the molecular mechanism of action of human proteins. The two-hybrid system has been used to identify putative interactions between proteins encoded by the genes of the Bcl-2 family [5,6]. Moreover, it was recently found that expression of Bax interferes with yeast cell growth. This unexpected inhibition of growth is prevented by coexpression of the anti-apoptotic genes Bcl-2 or Bcl-x_L. However, growth arrest induced by Bax expression is not associated with any apoptosis-associated characteristics, such as oligonucleosomal DNA-fragmentation [7]. Importantly, it was found that the presence of fully active mitochondria is required for both the deleterious effect of Bax and the protective effect of Bcl-2 [8].

It is suspected that mitochondria play a crucial role in the effects of the Bcl-2 family of genes in mammalian cells. When overexpressed, Bcl-2 is localized, although not exclusively, to the outer mitochondrial membrane [9]. Different hypotheses, based on observations made in diverse model systems, have been put forward to explain the role of mitochondria in apoptosis. One hypothesis involves the so-called permeability transition pore, a large mitochondrial megachannel whose opening is induced by Ca^{2+} and oxidative agents. Opening of the pore is able to release most molecules below 1500 daltons, including Ca^{2+} itself ([10], for a review), from the inner mitochondrial matrix which then leads to apoptosis. The drop in mitochondrial membrane potential ($\Delta\Psi$), caused by the opening of the system with pro-oxidants, is inhibited by cyclosporin A or bongkrekic acid thus preventing apoptosis [11]. Another set of observations involves cytochrome c, a soluble protein loosely bound to the outer face of the inner mitochondrial membrane. Recent evidence from two different groups show that release of cytochrome c to the cytosol is required for activation of the protease CPP32, an event that probably occurs in all cells undergoing apoptosis [12,13]. Moreover, it has been shown that Bcl-2 acts on mitochondria to prevent cytochrome c release. A third hypothesis involves Raf-1, a kinase of the *ras* family, which is targeted by Bcl-2 to the mitochondria. Mitochondria-associated Raf-1 probably phosphorylates the pro-apoptotic protein BAD (another Bcl-2 family protein) or other protein substrates to protect cells from apoptosis [14].

In this communication, we characterize the Bax-induced growth arrest phenotype in yeast cells at the mitochondrial level. We find that Bax expression stimulates the release of cytochrome c to the cytosol and causes a concomitant decrease in the amount of cytochrome c oxidase complex. However, the bc_1 complex and the F_0F_1-ATPase are not affected. Interestingly, release of cytochrome c and decrease in the amount of cytochrome c oxidase can be nearly completely prevented by coexpression of Bcl-x_L.

2. Materials and methods

The construction of yeast strains expressing Bax and Bcl-x_L under the control of *GAL10* promoter has been described previously [8]. Briefly, a construction carrying a chemically synthesized human *bax* gene (with yeast codon bias) fused to a *GAL10* promoter and a *SUC2* transcription terminator fragment was integrated at the *LEU2* locus of the wild-type strain HT444 (*MATa, leu2-3, leu2-112, his4-519, ura3, lys2*). The strain obtained after integration is referred to as Bi (i.e. Bax integrant). Bi was transformed with a CEN-plasmid encoding the Bcl-x_L gene that is also fused to the *GAL10* promoter and the *SUC2* terminator. The CEN-plasmid carries the *URA3* gene as yeast selection marker. The transformed strain is referred as Bi/Bcl-x_L. In all

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PII S0014-5793(97)01087-9

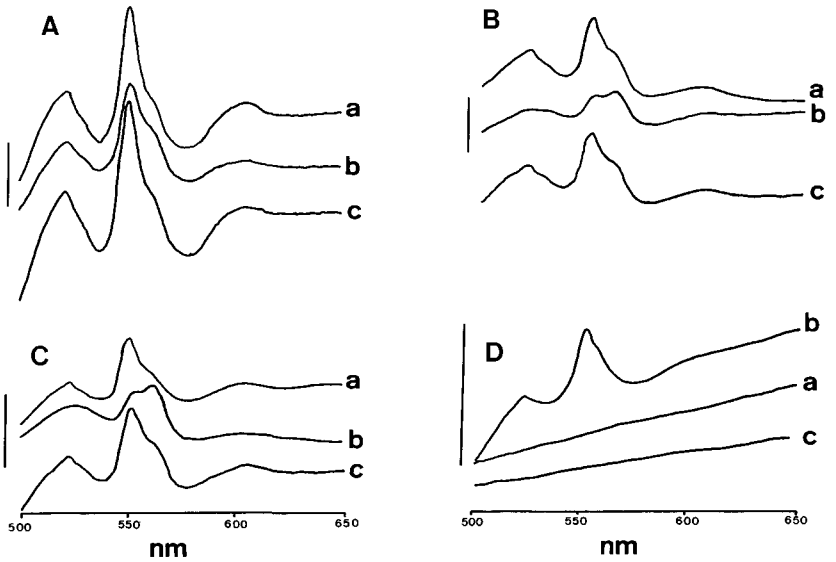

Fig. 1. Cytochrome spectra on whole cells (A), isolated mitochondria (B), and on the pellet (C), supernatant (D) of permeabilized spheroplasts. A: Cells were grown in YP 2% lactate medium until mid-exponential growth phase and then 0.5% galactose was added. Cells were harvested and washed twice with water. The cell suspension ($OD_{550nm} = 30$) was then displayed in the cuvettes of an Aminco DW2000 spectrophotometer and the dithionite-reduced vs. H_2O_2-oxidized spectra were recorded. B: Mitochondria were isolated from cells grown as described under Table 1. C, D: Mitochondria suspension was adjusted at 2 mg/ml. Cells were grown until late-exponential growth phase in a YP medium supplemented with 0.5% lactate. Then 0.25% galactose was added and cells were allowed to grow for another 4 h. Spheroplasts were prepared according to [16]. Spheroplasts were diluted to 5 mg/ml in a 10 mM potassium phosphate buffer (pH 6.8) containing 1 M sorbitol, 2 mM EGTA, 0.1% BSA. After addition of 50 μg nystatin/mg, spheroplasts were incubated for 10 min at 28°C. After a 3 min centrifugation at 12 000×*g*, cytochrome spectra were recorded both on the supernatant (D) and on the pellet resuspended in 1 ml water (C). For all recordings, the vertical bar represents 0.02 OD units. a, HT444; b, Bi; c, Bi/Bcl-x_L.

experiments, HT444, Bi and Bi/Bcl-x_L were pre-grown in minimal medium (containing yeast nitrogen base) with 2% glucose as carbon source and auxotrophic requirements. The cells were washed and transferred into complete medium (YP) that contained 1% yeast extract, 0.5% gelatin hydrolysate, 0.1% potassium phosphate, 0.12% ammonium sulfate, 0.5% or 2% (DL)-lactate, pH 5.0 with NaOH,

Table 1
Cellular and mitochondrial bioenergetic parameters

	HT444	Bi	Bi/Bcl-x_L
$ODmax_{550nm}$ in YNB lact. 2%	3.6	4.1	3.5
$ODmax_{550nm}$ in YNB lact. 2%+gal. 0.5%	7.7	1.4	7.1
$ODmax_{550nm}$ in YP lact. 2%	11.8	11.6	12.3
$ODmax_{550nm}$ in YP lact. 2%+gal. 0.5%	15.1	5.8	15.7
cytochrome b (pmol/mg)	444 ± 50	339 ± 42	437 ± 48
cytochromes $a+a_3$ (pmol/mg)	90 ± 12	6 ± 6	45 ± 15
cytochromes $c+c_1$ (pmol/mg)	602 ± 42	197 ± 21	569 ± 53
NADH → O_2 (state 4) (nat.O/min/mg)	348 ± 12	88 ± 17	404 ± 21
NADH → O_2 (state 3) (nat.O/min/mg)	749 ± 24	84 ± 10	768 ± 31
Ethanol → $Fe(CN)_6$ (nmol/min/mg)	840 ± 82	320 ± 25	765 ± 80
Ethanol → cytochrome c (nmol/min/mg)	102 ± 13	129 ± 5	129 ± 8
ATPase activity (nmol/min/mg)	800 ± 26	806 ± 18	798 ± 20
% oligomycin sensitivity	60 ± 12	49 ± 10	45 ± 7

Cell growth: Cells were inoculated into YNB or YP liquid media supplemented with 2% lactate and grown until 0.5–0.7 OD_{550nm} units. Then 0.5% galactose was added or not and cultures were let grown until reaching maximal OD_{550nm}. The error on this value was less than 0.2 OD_{550nm} units.
Mitochondrial bioenergetic parameters: Cells were grown in YP medium supplemented with 2% lactate until late-exponential growth phase, 0.5% galactose was added and cells were allowed to grow for 4 additional hours. Mitochondria were then isolated as described in [15]. All measurements (except ATPase) were made in a 10 mM Tris-maleate buffer (pH 6.8) containing 0.6 M mannitol, 2 mM EGTA and 0.3% BSA.
For cytochromes: Mitochondria were suspended at 4 mg/ml and the dithionite-reduced vs. H_2O_2-oxidized spectra were recorded in an Aminco DW2000 spectrophotometer.
For NADH → O_2: Mitochondria were suspended at 0.67 mg/ml in the buffer containing 5 mM phosphate and oxygen consumption was monitored with a Clark-type electrode; 2 mM NADH was used as the respiratory substrate; state 3 was obtained after addition of 1 mM ADP.
For ethanol → $Fe(CN)_6$: Mitochondria were suspended at 0.5 mg/ml in the presence of 1 mM potassium ferricyanide and 1 mM potassium cyanide. The reduction of ferricyanide by 20 mM ethanol was measured as the change of absorbance at 420 nm minus 470 nm.
For ethanol → cytochrome c: Mitochondria were suspended at 0.25 mg/ml in the presence of 13 μM yeast cytochrome c and 1 mM potassium cyanide. The reduction of cytochrome c by 20 mM ethanol was measured as the change of absorbance at 550 nm minus 540 nm.
For ATPase activity: Mitochondria (1 mg/ml) were suspended in a 0.2 M KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 8.4). The reaction was started by the addition of 5 mM ATP and stopped by the addition of 0.3 M trichloroacetic acid. After centrifugation, phosphate was measured in the supernatant by a colorimetric method. Oligomycin was added at 10 μg/mg protein.
All data in this table were obtained on a single mitochondria preparation for each strain and were reproducibly obtained from three different mitochondria preparations (two for HT444).

and were grown until late-exponential growth phase. The expression of Bax or Bax/Bcl-x_L was induced by growth of cells for 4 h after addition of 0.25% or 0.5% galactose.

Mitochondria were isolated as described in [15]. Spheroplasts were prepared and permeabilized as described in [16]. Proteins were measured with the biuret method. Spectra of cytochromes were recorded in an Aminco DW2000 double-beam spectrophotometer on dithionite-reduced vs. H_2O_2-oxidized samples. Oxygen consumption, ATPase activity and bc_1 complex activity are detailed in the legend of Table 1.

All reagents were obtained from Sigma/Aldrich.

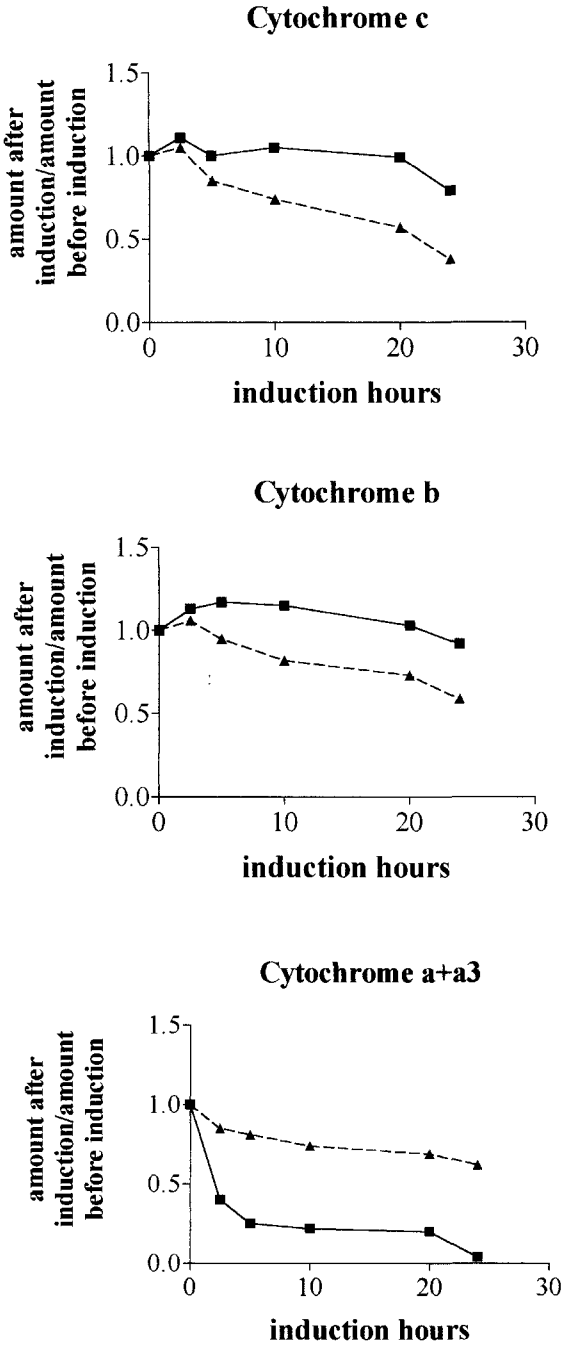

Fig. 2. Amounts of cytochromes in Bi (squares) and Bi/Bcl-x_L (triangles) cells following induction in galactose. Similar conditions as in Fig. 1A were used. Cytochrome c, cytochrome b and cytochrome $a+a_3$ were quantified by the difference in absorbance at 550 nm minus 540 nm, 561 nm minus 575 nm and 603 nm minus 630 nm and expressed as the ratio of the amount of cytochromes after galactose induction over the amount of cytochromes before induction. Results are representatives of three similar experiments.

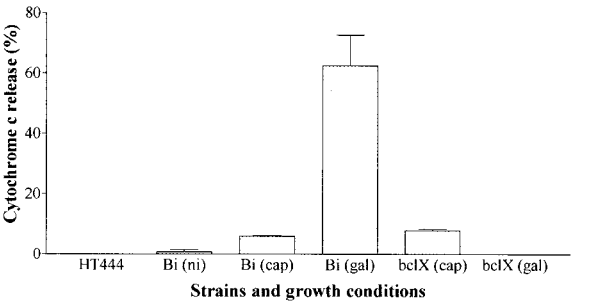

Fig. 3. Cytochrome c release in permeabilized spheroplasts. Cells were grown in YP medium supplemented with 0.5% lactate until late-exponential growth phase. Then 0.25% galactose or 5 g/l chloramphenicol was added. Measurements of cytochromes were performed as described under Fig. 1C, D. Results represent the % of cytochrome c found in the supernatant. Each value is means ± S.D. of 3 to 6 independent preparations. HT444: wild-type grown in the absence of galactose; Bi(ni): Bi grown in the absence of galactose; Bi(cap): Bi grown in the presence of chloramphenicol; Bi(gal): Bi grown in the presence of galactose; Bcl-x(cap): Bi/Bcl-x_L grown in the presence of chloramphenicol; bclX(gal): Bi/Bcl-x_L grown in the presence of galactose.

3. Results and discussion

The phenomenon of growth arrest induced by Bax expression is illustrated in Table 1, where the maximal OD reached by the cultures of the wild-type strain HT444, the Bax-expressing strain and the Bax/Bcl-x_L-coexpressing strain are reported. It appears clearly that expression of Bax completely stops the growth of yeast cells. Cell growth is restored again by coexpression of Bcl-x_L.

Cytochrome spectra were recorded on whole cells to depict alterations of the respiratory chain (Figs. 1A and 2). It appears that expression of Bax induces a decrease in the amount of cytochromes $a+a_3$ whereas levels of cytochromes b and $c+c_1$ were not similarly affected. It should be noted that, although the coexpression of Bcl-x_L prevents the disappearance of cytochromes $a+a_3$, it does not fully restore the normal amount of cytochrome $a+a_3$ found in wild-type cells (this appears more clearly on isolated mitochondria, see below).

Mitochondria were isolated from cells grown on a medium supplemented with 2% (DL)-lactate until the late-exponential growth phase (OD_{550nm} = 2–2.5). 0.5% galactose was added and cells were grown further for another 4 h. Recordings of cytochrome on mitochondria (Fig. 2B) confirmed the almost complete disappearance of cytochrome $a+a_3$ and insensitivity of cytochrome b to Bax expression. But an additional phenomenon was evidenced: mitochondria from cells expressing Bax lost most of their cytochrome c (Fig. 1B and Table 1). This did not occur in mitochondria isolated from cells coexpressing Bax and Bcl-x_L, whereas, as stated above, the amount of cytochrome $a+a_3$ was significantly reduced as compared to the wild-type strain.

We measured the incidence of these alterations on the activity of the respiratory chain. As expected, mitochondria isolated from cells expressing Bax presented a dramatically reduced oxygen consumption with both external (NADH; Table 1) and internal (ethanol; not shown) electron donor. Respiration was so limited in mitochondria isolated from Bax-expressing cells that it was not stimulated anymore by ADP. Similarly, the reduction of ferricyanide by ethanol in the presence of cyanide (to inhibit cytochrome c oxidase) was

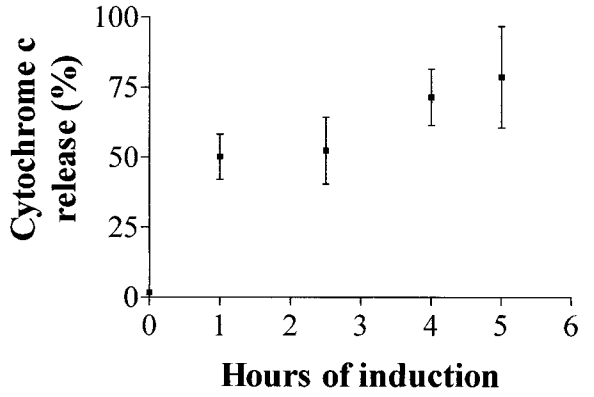

Fig. 4. Kinetics of cytochrome c release in Bax-expressing cells. Similar conditions as in Fig. 3.

markedly reduced, showing that reduced levels of cytochrome c per se results in a decrease of the electron transfer rate. On the other hand, the rate of electron transfer from ethanol to (exogenous) cytochrome c was not altered, thus confirming the absence of alteration of the bc_1 complex.

We also measured the activity of the oligomycin-sensitive F_0F_1-ATPase to confirm that the lack of stimulation of respiration by ADP was not caused by an alteration of this complex. The ATPase activity and its degree of sensitivity to oligomycin (reflecting the attachment of the catalytic F_1 part to the membrane sector F_0) were unaffected by Bax expression (Table 1).

Since the mechanical treatments during isolation may have affected mitochondria, we investigated whether this release of cytochrome c also occurred in whole cells. For this, we prepared spheroplasts, and then permeabilized them with nystatin under conditions known to release cytosolic enzymes but which also allow the maintenance of the integrity of intramembrane structures such as the mitochondria [16]. We then looked for the presence of cytochrome c in the supernatant after centrifugation of cells. Results of these experiments are depicted in Fig. 1C, D and Fig. 3. It appears clearly that a large proportion of cytochrome c in Bax-expressing cells is now released by nystatin, indicating its cytosolic localization. This release is very rapid, since only after 1 h of induction 50% of the cytochrome c is already in the cytosol (Fig. 4). None or negligible amounts of cytochrome c were released in the supernatant of wild-type cells, non-induced Bax-expressing cells, or cells coexpressing Bax and Bcl-x_L. We compared the effect of Bax-expression to the effect of chloramphenicol, an inhibitor of mitochondrial protein synthesis to investigate the possibility of a relationship between cytochrome c oxidase reduction and cytochrome c release. Although chloramphenicol did induce the release of some cytochrome c (only 10–15%), this was far from the amplitude of the phenomenon observed in Bax-expressing cells.

4. Conclusion

Data reported in this paper allow us to draw a correlation between Bax-induced growth arrest of yeast cells and two major alterations in the respiratory chain.

First, we observe a dramatic decrease of the amount of cytochrome $a+a_3$ and thus of the cytochrome c oxidase complex. The latter could be explained by the fact that assembly of the complex requires the attachment of both hemes to one of the three mitochondrial DNA-encoded subunits. The decrease in the amount of this complex is, however, not related to a general problem in mitochondrial DNA-encoded protein synthesis since activities of the bc_1 complex and the ATPase complex (bc_1 has one while ATPase has three subunits encoded by mitochondrial DNA) are not altered.

Second, we observe a release of cytochrome c from mitochondria to the cytosol. Although they occur simultaneously, this second phenomenon does not appear to have an obvious correlation to the first one since chloramphenicol-induced inhibition of mitochondrial protein synthesis does not have a profound effect on the release of cytochrome c.

Whatever the mechanism underlying Bax-induced cytochrome c release from mitochondria, it is of special interest since such a release occurs in mammalian cells undergoing apoptosis and might be the key to the cascade of biochemical events that drive cells to programmed cell death [12,13]. Additionally, we can draw a parallel between the prevention of cytochrome c release by Bcl-2 in mammalian cells and the prevention by Bcl-x_L that we observe in yeast cells. This means that, although yeast cells do not have the 'genetic background' required for the late steps of apoptosis (Bax expression in yeast does not induce any apoptosis-associated characteristic such as DNA-fragmentation [7]), yet the expression of Bax is able to elicit the early steps of apoptosis. Yeast may therefore be a suitable model for studying the molecular mechanism of the action of genes of the Bcl-2 family.

Acknowledgements: This work is supported by grants from the Centre National de la Recherche Scientifique and the Conseil Régional d'Aquitaine.

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