A cytochrome *c*–GFP fusion is not released from mitochondria into the cytoplasm upon expression of Bax in yeast cells

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Abstract To study Bax-induced release of cytochrome *c* in vivo, we have expressed a cytochrome *c*–GFP (green fluorescent protein) fusion in *Saccharomyces cerevisiae* cells null for the expression of the endogenous cytochrome. We show here that cytochrome *c*–GFP is efficiently localised to mitochondria and able to function as an electron carrier between complexes III and IV of the respiratory chain. Strikingly, while natural cytochrome *c* is released into the cytoplasm upon expression of Bax, the cytochrome *c*–GFP fusion is not. Nevertheless, cells co-expressing Bax and the cytochrome *c*–GFP fusion die, indicating that mitochondrial release of cytochrome *c* is not essential for cell death to occur in yeast. The failure to release cytochrome *c*–GFP is presumed to arise from increased bulk due to the GFP moiety. We propose that in intact yeast cells, Bax-induced release of cytochrome *c* into the cytoplasm occurs through a selective pore and not as a consequence of the non-specific breakage of the mitochondrial outer membrane.

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Key words: Cytochrome *c*; Green fluorescent protein; Bax; Yeast; Cell death; Mitochondrion

1. Introduction

Because they release apoptogenic factors, mitochondria play a key role during programmed cell death in multicellular organisms [1]. Cytochrome *c* was the first mitochondrial apoptogenic protein identified and its translocation from the intermembrane space (IMS) to the cytoplasm has been observed in many models of apoptosis [2]. Another mitochondrial apoptogenic factor was recently identified, the apoptosis-inducing factor (AIF), which induces apoptotic changes such as chromatin condensation and exposure of phosphatidylserine in the plasma membrane [3]. In some cell types, procaspases-2 and -9 are also confined in the intermembrane space and are released at the same time as cytochrome *c* and AIF [4].

Proteins of the mammalian Bcl-2 family are either anti-apoptotic (such as Bcl-2 and Bcl-x_L) or pro-apoptotic (such as Bax and Bak). Cell death can be induced in yeast by expressing a pro-apoptotic member of this family [5,6]. Moreover, the expression of Bax in yeast cells triggers similar apoptotic changes to those occurring in mammalian cells [7]. Mitochondria also seem to be involved in Bax-induced cell death in yeast since, as in mammalian cells, a release of cytochrome *c* is observed [8].

A central role for cytochrome *c* and AIF in the apoptotic pathway has been highlighted by studies showing that injection of these two effectors of cell death into the cytosol of mammalian cells induced apoptosis [3,9]. In the mammalian cytoplasm, cytochrome *c* associates with the monomeric apoptotic protease activating factor-1 (Apaf-1). Subsequent hydrolysis of ATP/dATP promotes oligomerisation of Apaf-1. The resultant multimeric complex, the apoptosome, recruits and activates procaspase-9 [10,11]. Once activated by cleavage, mature caspase-9 can then process procaspase-3, setting into motion the terminal caspase cascade [12].

The mechanism of release of mitochondrial apoptogenic factors is still unclear. Based on in vitro experiments, three hypotheses have been proposed [13]. First, when mitochondria are induced to swell, the release of IMS components occurs. Evidence for this idea comes from the induction of the non-specific permeability transition pore which correlated with mitochondrial matrix swelling. The consequent disruption of the outer mitochondrial membrane led to the release of proteins from the IMS such as cytochrome *c* [14,15]. Second, Bax itself can form very large channels in planar lipid bilayers. Should such channels form in the outer mitochondrial membrane, then they might be large enough to permit passage of cytochrome *c* [16]. Third, Bax has been shown to interact with the voltage dependent anion channel (VDAC) and the adenine nucleotide carrier (ANT) [17]. Since VDAC is essential for cytochrome *c* release in both wild type and VDAC-deficient yeast mitochondria [17], Bax could complex with VDAC and/or ANT to form a channel for cytochrome *c* release [13].

The requirement for a specific mechanism of cytochrome *c* release can be questioned since, in addition to proteins that have a specific role in apoptosis (i.e. cytochrome *c*, AIF, procaspases-2 and -9), other proteins from the IMS are also translocated into the cytoplasm (e.g. adenylate kinase, sulfite oxidase) [18]. Based on in vitro studies, it has been proposed that protein release involves a general alteration of the permeability of the outer mitochondrial membrane, both in mammalian [15] and in yeast [19] cells. However, there is no indication that in vivo, a generalised translocation into the cytoplasm of IMS proteins takes place.

We have designed experiments to probe the mechanism of the release of cytochrome *c* in vivo. We have generated a fusion between cytochrome *c* and the green fluorescent protein (GFP) and expressed this fusion in yeast cells null for the expression of natural cytochrome *c*. This enables efficient delivery of the fusion to mitochondria. Confocal laser scanning microscopy (CLSM) and Western blotting techniques are used to show that this cytochrome *c*–GFP fusion is not released from mitochondria on expression of Bax in intact yeast cells.

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2. Materials and methods

2.1. Plasmid construction and yeast strains

A DNA cassette encoding yeast cytochrome *c* (isoform 1, Cyc1p) was amplified by polymerase chain reaction (PCR) and cloned into the *Bgl*II and *Not*I sites of the yeast multicopy expression vector pAS1N [20], to form pAS1N–Cyc1p. pAS1N–Cyc1p–GFP was constructed as follows. A second DNA cassette encoding Cyc1p was amplified by PCR using oligonucleotide primers designed such that nucleotides at the 3′ end specifying the stop codon of cytochrome *c* were removed. This cassette was then cloned into the *Bgl*II site of pAS1N–GFP [20]. The correct orientation of the Cyc1p-encoding insert relative to the promoter and GFP encoding sequence was checked by PCR.

The expression vector pCM189 carrying the human *bax* gene under the control of a tetracycline-regulatable promoter has been described previously [21]. A DNA sequence encoding the c-*myc* epitope was introduced at the 3′ end of the *bax* gene [5].

The yeast strain B-06748 (*MAT*a, *his3*, *leu2*, *trp1*, *ura3*, *cyh2*, *cyc1::lacZ*, *cyc7::CYH2*), null for the expression of both isoforms 1 and 7 of cytochrome *c* (Cyc1p and Cyc7p, respectively) was transformed with the vector pAS1N–Cyc1p or pAS1N–Cyc1p–GFP. These vectors carry the *LEU2* gene as a selectable marker enabling the selection of transformants on synthetic minimal medium (0.7% yeast nitrogen base w/o amino acids (Difco), 2% glucose) (SD), supplemented with histidine, tryptophan and uracil (each at 0.1 mg/ml). Cells carrying pAS1N–Cyc1p or pAS1N–Cyc1p–GFP were subsequently transformed with pCM189-*bax* (which carries the *URA3* selectable marker) and transformants were selected on minimal medium supplemented with histidine and tryptophan.

2.2. Growth conditions and preparation of spheroplasts

Cells were pregrown in SD medium with relevant auxotrophic requirements, containing doxycycline (1 μg/ml) to prevent Bax expression [22]. The cells were then washed and 2×10^7 cells inoculated into SaccE medium containing 2% ethanol as a carbon source [23] containing auxotrophic requirements, in the presence (Bax-repressed) or in the absence (Bax-induced) of doxycycline. The increase of turbidity of cultures was followed using a Klett–Summerson photoelectric colorimeter.

Survival tests were performed as described [21].

For spheroplast preparation, cells were pregrown as above. Cells were harvested, washed and inoculated into fresh SaccE medium either with (Bax-repressed) or without (Bax-induced) doxycycline. Cells were then allowed to grow overnight and harvested in the mid-log-phase of growth. Spheroplasts were prepared according to Averet et al. [24] with the modification that no bovine serum albumin (BSA) was included in buffer S (1 M sorbitol, 2 mM MgSO$_4$, 0.5 mM EGTA, 10 mM K$_2$HPO$_4$, pH 6.8).

2.3. Permeabilisation of spheroplasts and protein analysis

Ten mg dry weight spheroplasts were suspended in 1 ml of buffer S. After the addition of 0.25 mg nystatin (2.5 mg/ml in distilled water), the suspension was incubated at 28°C for various times. Spheroplasts were then centrifuged in a microfuge for 3 min and the supernatant and pellet separated. Pellets were suspended in 1 ml distilled water. Proteins from the pellet and the supernatant were precipitated by the addition of 0.1 ml 3 M trichloroacetic acid. After centrifugation, proteins from the pellets were solubilised in 0.2 ml 5% sodium dodecyl sulfate (SDS). Dissociation buffer (0.2 ml) was then added and 50 μg protein separated by electrophoresis on 15% SDS–polyacrylamide gel. Proteins from the supernatants were solubilised in 50 μl 5% SDS and 50 μl dissociation buffer and 10 μl portions electrophoresed as above. Proteins were then transferred to Immobilon P membranes (Millipore, MA, USA). Membranes were incubated with rabbit polyclonal antibodies directed against cytochrome *c* diluted 1/2000 (kindly provided by Dr F. Sherman, University of Rochester). Immunoblotting and estimation of the intensity of the signals were performed as previously described [25].

For whole cell lysates, yeast cells were lysed [26], and Western analysis was carried out as described above, with antibodies directed against cytochrome *c*. Bax expression was analysed [21] with monoclonal antibodies directed against the c-*myc* epitope diluted 1/500 (gift from Dr C.A. Mitchell, Monash University).

Protein concentrations were determined according to Lowry et al.

[27] in the presence of 5% SDS. BSA was used as the standard protein.

2.4. Citrate synthase (CS) and glucose-6-phosphate dehydrogenase (G6PDH) activities

The CS activity was measured according to Priault et al. [19]. The G6PDH activity was measured as the formation of NADPH. An aliquot of the samples (50 μl) was added to 0.9 ml 50 mM triethanolamine/HCl buffer (pH 7.4) containing 3.75 mM EDTA, 7.5 mM MgCl$_2$ and 1 mM NADP. The absorbance was measured at 340 nm in a Beckman DU7500 spectrophotometer ($\varepsilon = 6200$ M^{-1} cm^{-1}).

2.5. CLSM

Yeast cells were pregrown in SD medium and then cultured in SaccE medium in the presence or absence of doxycycline as described in Section 2.2. Cells were harvested in the mid-log phase, washed in distilled water and air-dried onto the under-side of poly-L-lysine-coated coverslips. CLSM images were generated using the Leica TCS NT system (Leica Instruments, Australia), FITC filter sets and illumination with a 488 nm laser line [20].

3. Results and discussion

3.1. The cytochrome c–GFP fusion protein is functional

We found that cells with endogenous cytochrome *c* genes, transformed with pAS1N–Cyc1p–GFP were unable to accumulate the Cyc1p–GFP fusion efficiently or specifically in mitochondria (data not shown). Therefore, to prevent expression of any endogenous cytochrome *c*, cells null for the expression of the two isoforms Cyc1p and Cyc7p were used in all experiments reported here. Cells of strain B-06748 are unable to grow on a non-fermentable substrate such as ethanol since they lack a respiratory chain function. These cells were transformed with expression vectors such that cells would express either Cyc1p or Cyc1p–GFP, in each case combined with the inducible expression of Bax. Transformants were selected on appropriate medium containing glucose as a substrate (plus doxycycline to prevent the expression of Bax). Growth of the cells was then tested on ethanol-containing medium (Fig. 1). In the presence of doxycycline, cells expressing Cyc1p–GFP showed substantial growth, although the rate was slightly decreased relative to cells expressing Cyc1p (Fig. 1A,B, open circles). The Cyc1p–GFP fusion is evidently able to function as a mobile electron carrier between complexes III and IV of the respiratory chain.

When cells were grown in the absence of doxycycline to activate Bax expression, growth rates were reduced but not fully abolished (Fig. 1A,B, closed circles), as has been previously observed by others [19]. This reduction in growth rate at a population level was due to Bax-induced mortality. Indeed, when aliquots of the cultures were diluted and plated to measure colony forming efficiency on non-selective doxycycline-containing plates, the number of colonies after Bax expression was reduced by 60–70% in both cases (Fig. 1C,D). This result suggested that the expression of Cyc1p–GFP did not modify the ability of Bax to induce cell death.

Lysates from cells expressing Cyc1p and Cyc1p–GFP were probed with polyclonal antibodies directed against Cyc1p (Fig. 2). Cyc1p migrated with a mobility corresponding to its size of 14 kDa (Fig. 2, lane 1). The size of the corresponding immunodetected band in lysates from cells expressing Cyc1p–GFP was about 45 kDa (Fig. 2, lane 3), which is consistent with that expected (14 kDa for Cyc1p plus 27 kDa for GFP). Significantly, no free cytochrome *c* was detected in this experiment, suggesting that the majority of the fusion Cyc1p–
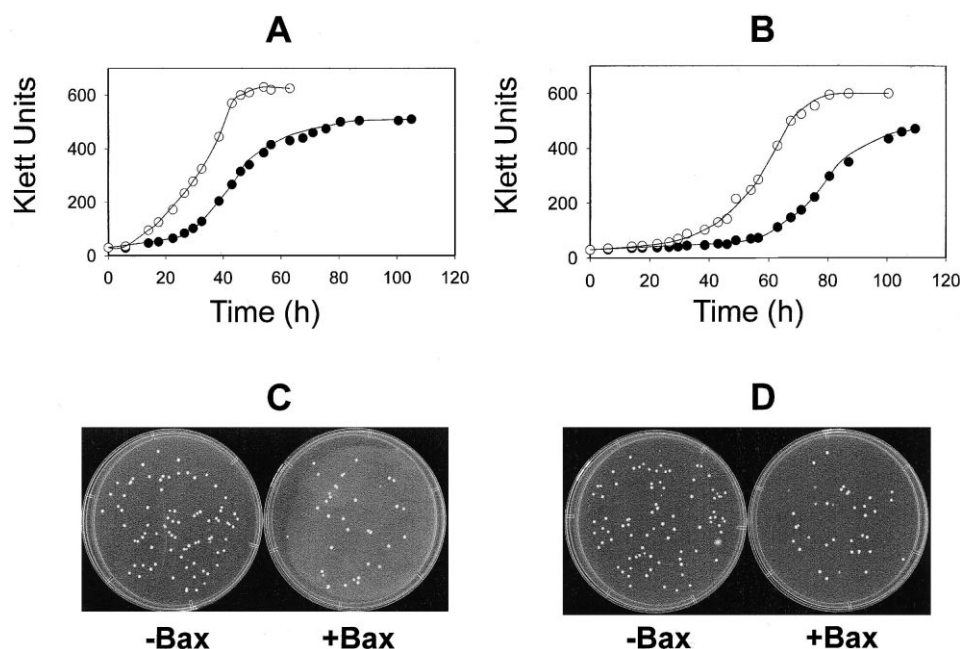
Fig. 1. Bax-induced cell lethality. A and C: Cells expressing Cyc1p. B and D: Cells expressing Cyc1p–GFP. Cultures were grown in the presence (open circles) or absence (closed circles) of doxycycline (see A and B). When the turbidity reached 400 Klett units, an aliquot of each culture was taken, diluted and spread at about 100 cells/plate on solid complete glucose medium in the presence of doxycycline. C and D: Plates from the (+doxycycline) culture (−Bax) and the (−doxycycline) culture (+Bax). C: (−Bax), 81 cells; (+Bax), 33 cells. D: (−Bax), 94 cells; (+Bax), 36 cells.

GFP remained intact after expression and import into the mitochondria. It is therefore unlikely that there would be enough free Cyc1p to sustain the growth of these cells on ethanol. Using antibodies specific for GFP, some free GFP was detected at 27 kDa (not shown), consistent with the very stable nature of GFP, highly resistant to protease degradation. Expression of Bax did not modify the expression of Cyc1p and Cyc1p–GFP (Fig. 2, lanes 2 and 4, respectively). Therefore, any effect of Bax cannot be attributed to an indirect effect on the level of cytochrome c in the cells.

The cellular distribution of Cyc1p–GFP was imaged using CLSM. The fluorescence displayed a filamentous array (Fig. 3A), consistent with a location within the mitochondria [20]. When cells were loaded with MitoTracker Red (CMXRos) which localises to mitochondria [28], GFP fluorescence displayed the same pattern as MitoTracker Red fluorescence (not shown). Taken together, these results showed that Cyc1p–GFP is a functional electron carrier and could be correctly imported into, and localised within, the mitochondrial IMS.

3.2. The cytochrome c–GFP fusion is not released upon expression of Bax

Cells were grown in the presence of doxycycline to repress Bax expression. Spheroplasts were prepared and the plasma membrane permeabilised with nystatin for various times as described in Section 2. In the first instance, permeabilisation of the plasma membrane with nystatin was calibrated using two enzyme markers: G6PDH, a cytosolic enzyme, and CS, a mitochondrial matrix enzyme. As shown in Fig. 4A, G6PDH activity was detected in the supernatant of spheroplasts after 5 min permeabilisation with nystatin. No CS activity was present in the supernatant. After 14 min incubation with nystatin, intracellular membranes, such as mitochondrial mem-

branes, were also permeabilised and CS activity in the supernatant increased substantially (Fig. 4A). On the basis of these results, cytochrome c present in the cytosol should be detected in the supernatant fraction prepared from spheroplasts permeabilised for 5 min with nystatin.

Cyc1p and Cyc1p–GFP were detected in the pellet fraction of non-permeabilised spheroplasts, whether or not Bax was expressed (Fig. 4B, compare lanes 1 and 7). Cytochrome c was barely detectable in the corresponding supernatants (Fig. 4B, lanes 2 and 8). When the experiment was repeated with permeabilised spheroplasts prepared from cells grown in
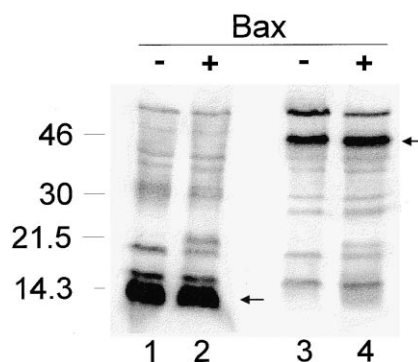

Fig. 2. Cyc1p and Cyc1p–GFP expression. Cell lysates were prepared as described in Section 2 and 50 μg of proteins were separated in a 15% polyacrylamide gel. After transfer, membranes were probed with polyclonal antibodies against cytochrome c. Lanes 1 and 3: lysates from cells expressing Cyc1p or Cyc1p–GFP, respectively, grown in the absence (−) of Bax expression. Lanes 2 and 4: lysates from cells expressing Cyc1p or Cyc1p–GFP, respectively, grown in the presence (+) of Bax expression. Molecular weight standards are indicated on the left side in kDa. Arrows indicate the position of Cyc1p (lanes 1–2) and Cyc1p–GFP (lanes 3–4).
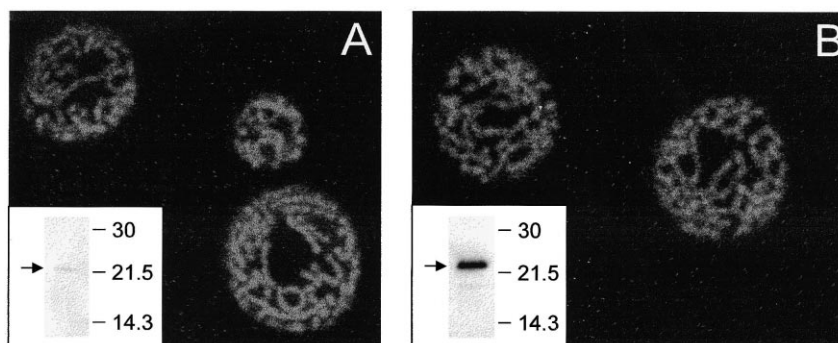
Fig. 3. CLSM images of cells expressing Cyc1p–GFP under conditions of the absence (A) or presence of Bax expression (B). Inset in each panel: expression of Bax (arrowed) was detected in cell lysates by Western blotting. Positions of molecular weight standards are indicated at the right (kDa).

the presence of doxycycline (Bax-repressed), Cyc1p and Cyc1p–GFP were associated with the pellet fraction (Fig. 4B, lane 3). No cytochrome c was detected in the corresponding supernatant fraction (Fig. 4B, lane 4). When the cells were grown in the absence of doxycycline (Bax-induced), Cyc1p was detected both in the pellet and supernatant fractions of permeabilised spheroplasts (Fig. 4B, lanes 9 and 10), indicating a release of Cyc1p into the cytoplasm. Surprisingly, Cyc1p–GFP was not detected in the supernatant (Fig. 4B, lane 10). It was concluded that Cyc1p–GFP was not released in the cytoplasm upon expression of Bax. When permeabilisation of the plasma membrane was conducted for 14 min, the integrity of mitochondrial membranes within cells was lost (as shown by the presence of CS in the supernatant fractions, Fig. 4A) and release of Cyc1p or Cyc1p–GFP was observed whether or not Bax was expressed (Fig. 4B, compare lanes 5 and 6 with 11 and 12).
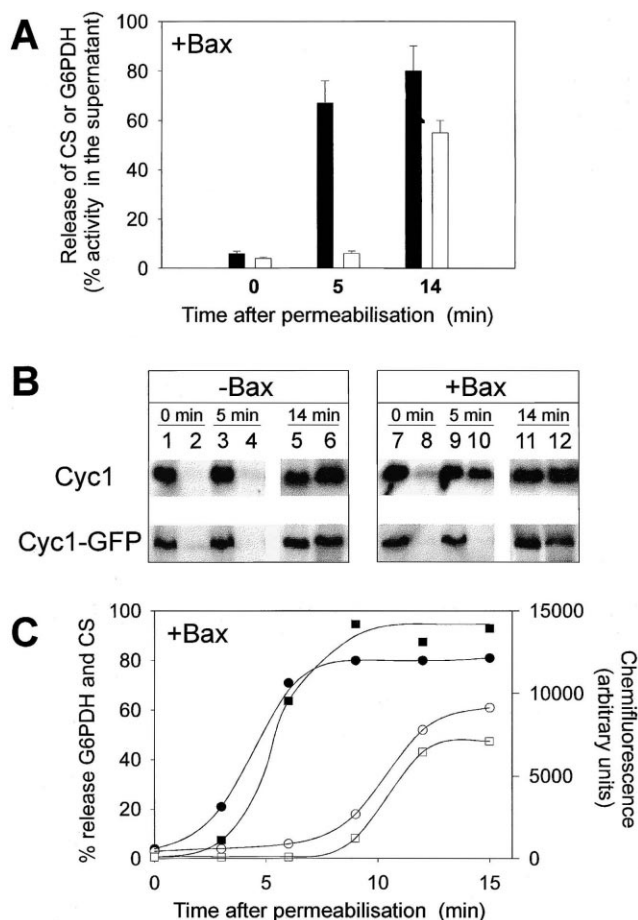
After permeabilisation with nystatin for various times, the presence of cytochrome c in the supernatant was probed in parallel with that of G6PDH and CS. Release of Cyc1p occurred at the same time as that of G6PDH (Fig. 4C). In contrast, release of Cyc1p–GFP paralleled that of CS (Fig. 4C). These results definitively confirmed that Cyc1p was released in the cytosol upon expression of Bax, but not Cyc1p–GFP.

Cells expressing Cyc1p–GFP were grown in the presence or absence of doxycycline and imaged by CLSM (Fig. 3). Under Bax-repressed conditions, the fluorescence was clearly associated with the mitochondria only (Fig. 3A) (see Section 3.1). Under Bax-induced conditions, Cyc1p–GFP fluorescence displayed the same pattern (Fig. 3B); no fluorescence could be observed in the cytoplasm, confirming the results shown in Fig. 4. The expression of Bax was confirmed by immunoblot (Fig. 3A,B, inset).

3.3. Conclusions

We have engineered in yeast, a non-releasable form of cytochrome c. The morphology of mitochondria, as judged by

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Fig. 4. Bax-induced cytochrome c release in permeabilised spheroplasts. Cells expressing Cyc1p or Cyc1p–GFP were grown in SaccE medium, until the mid-log phase in the presence (−Bax) or the absence (+Bax) of doxycycline. Spheroplast preparation and permeabilisation were performed as described in Section 2. A: CS (□) and G6PDH (■) activities were measured on non-permeabilised and permeabilised spheroplasts. Results are expressed as percentage of enzyme activity found in the supernatant before (0 min) or after (5 or 14 min) permeabilisation. B: Proteins from the pellet fraction (odd numbered lanes) and the supernatant fraction (even numbered lanes) were separated in a 15% SDS–polyacrylamide gel, transferred to Immobilon membrane and probed with cytochrome c polyclonal antibodies. The absence (−Bax) or presence (+Bax) of expression of Bax is indicated, together with the times of nystatin treatment (min) to permeabilise spheroplasts. C: Release of G6PDH (●), CS (○), Cyc1p (■) and Cyc1p–GFP (□) was assessed after permeabilisation of spheroplasts for various times. Results are expressed as percentage of CS and G6PDH activity in the supernatant, or intensity of chemifluorescence signal for Cyc1p and Cyc1p–GFP detected in the supernatant. 100% activity was 18 ± 2 nmol min^{-1} mg^{-1} protein and 46 ± 5 nmol min^{-1} mg^{-1} protein for CS and G6PDH, respectively (A and C). Similar results were obtained in three other independent spheroplast preparations.

the fluorescence of GFP in the IMS, was not modified after Bax expression. This confirmed previous observations based on electron microscopy images of Bax-expressing yeasts [21]. The absence of gross alteration in mitochondrial distribution or morphology in cells expressing Bax suggests that such changes were not responsible for the non-release of Cyc1p–GFP. Moreover, despite the absence of translocation of Cyc1p–GFP into the cytosol, the expression of Bax still induced cell death. There are two main conclusions arising from these results.

First, the release of cytochrome *c* is not required for Bax-induced cell death to occur in yeast cells. It was shown that in cells unable to express cytochrome *c*, Bax-induced lethality was not prevented [21]. However, the only source of energy for the growth of those cells deprived of cytochrome *c* due to gene knockout is from glycolysis. It could be argued that cytochrome *c* is not essential for Bax-induced lethality under fermentative (but not respiratory) conditions. Here, we show that even under respiratory conditions, the release and the presence of cytochrome *c* in the cytoplasm is not essential for Bax-induced cell death in yeast. Bcl-2 proteins regulate cytochrome *c* release both in mammalian [13] and yeast [19] cells. However, contrary to mammalian cells, yeast do not express caspases, which could explain why the presence of cytochrome *c* in the yeast cytoplasm is not essential for cell death.

Second, these results suggest that in vivo, Bax does not induce a non-specific rupture of the mitochondrial outer membrane and a concomitant release of any proteins of the IMS. Indeed, GFP has been shown to diffuse freely in the cytoplasm and in intracellular organelles [29]. Thus, it is unlikely that GFP would prevent the diffusion of cytochrome *c* into the cytoplasm upon disruption of the outer membrane. There are two main reasons why Cyc1p–GFP might not show release. Either the protein fusion is too big to permit passage through a pore or the C-terminal domain of Cyc1p to which GFP is fused must be free for cytochrome *c* to be released. However, the presence of a hexahistidine tag at the C-terminus of Cyc1p did not prevent its release induced by Bax (not shown). It is, therefore, more likely that Cyc1p–GFP is not translocated in the cytoplasm because of its size. If so, it provides evidence in support of the translocation of cytochrome *c* through a channel, as proposed by some authors [30]. Indeed, in order for the assembly of the apoptosome to be efficient, one may consider that the release of cytochrome *c* would be regulated both in time and in space and, therefore, occurs by a specific mechanism.

Release of a cytochrome *c*–GFP fusion was observed in mammalian cells [31]. However, in this study, apoptosis was induced by staurosporine. Therefore, either the mechanism of the release of cytochrome *c* is different in mammalian cells from yeast cells or the mechanism varies according to the apoptosis inducer.

The possibility of engineering non-releasable forms of cytochrome *c* in yeast opens a new avenue for the elucidation of the mechanism of cytochrome *c* release during cell death. It would now be important to define size limits for release and the nature of the release apparatus.

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