Cell Death and Differentiation (1997) 4, 263–271

Expression of bak in *S. pombe* results in a lethality mediated through interaction with the calnexin homologue Cnx1

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Received 14.10.96; revised 12.12.96; accepted 16.12.96
Edited by M. Piacentini

Abstract

Expression studies in the yeast *S. pombe* have been utilised to establish the basis for a genetic analysis designed to identify the lethal partners of the pro-apoptotic proteins bak and bax. Bak expression in *S. pombe* is lethal and this lethality is rescued by co-expression of bcl-2 or bcl-x_L. *S. pombe* cells expressing bak have a terminal phenotype in which the majority of cells are blocked in the G1 phase of the cell cycle while the remainder of cells, unable to complete M-phase, mis-coordinate the timing of subsequent events in the cell cycle. Although bax expression in *S. pombe* gives rise to a slow growth phenotype, not a lethality, bax expressing cells display the same cell cycle phenotypes described for bak. Electron microscopy of cells expressing bak reveals a dramatic accumulation of large vesicular structures. A two-hybrid screen designed to identify *S. pombe* proteins which interact with bak, isolated the *S. pombe* calnexin homologue *cnx1*. Genetic analysis demonstrates that the Cnx1 domain which binds to bak in two-hybrid experiments, is necessary for bak lethality in *S. pombe*. This report identifies a lethal interacting partner for bak and the observations suggest a model for bak mediated lethality which can be tested in higher cells.

Keywords: apoptosis, programmed cell death, Schizosaccharomyces pombe, bcl-2 family, bak, calnexin

Abbreviations: MM: minimal medium; NLM: nitrogen limiting medium; FACS: fluorescence activated cell sorting

Introduction

In higher eukaryotic cells an important control of the apoptotic programme is exerted by members of the bcl-2 family of proteins (Williams and Smith 1993; Reed 1994; Oltvai and Korsmeyer 1994). Different members of this family, on the basis of over-expression or gene knockout experiments, have the ability to commit the cell to, or protect the cell from, apoptosis (Garcia *et al*, 1992; Oltvai *et al*, 1993; Veis *et al*, 1993; Motoyama *et al*, 1995). Proteins of the bcl-2 family demonstrate a common ability to form homo and hetero-dimers, thus leading to a model where a cell's apoptotic fate is determined by competitive binding between lethal and protecting members of the family (Korsmeyer *et al*, 1993). However, the complexity of interactions amongst members of the bcl-2 family (Farrow and Brown 1996), coupled with the lack of any known biochemical activity for these proteins has hampered the identification of other components of the apoptotic pathway in higher cells. Consequently, fundamental questions remain concerning their mechanisms of action, in particular the identity of the cellular targets for the two known apoptotic inducers bak (Farrow *et al*, 1995; Chittenden *et al*, 1995; Kiefer *et al*, 1995) and bax (Oltvai *et al*, 1993).

Bcl-2 family member homologues have been identified in evolutionarily distinct species suggesting that, at least at the level of these proteins, the apoptotic programme has been conserved throughout evolution. Indeed, only the genetic analysis of somatic cell death in the nematode *C. elegans* has established a linear apoptotic pathway involving a bcl-2 family member (Hengartner and Horvitz 1994; Ellis *et al*, 1991). Until recently, it was widely believed that the evolution of an 'altruistic' apoptotic pathway coincided with the emergence of multi-cellular organisms. This supposition however, failed to appreciate that many features regulated by apoptosis in multicellular organisms (i.e. cell number, differentiation state and growth arrest) are important features of the life cycle of many unicellular eukaryotes. Recently, several reports have indicated that an apoptosis-like process operates in several different unicellular eukaryotes, thus raising important new questions concerning the evolutionary origin of apoptosis (Cornillon *et al*, 1994; Ameisen *et al*, 1995; Wellburn *et al*, 1996, reviewed in Ameisen 1996).

These new insights have introduced the possibility that unicellular eukaryotes may be of utility as genetically tractable models for apoptotic death. Assessing the contribution that such a model could make however, may only be possible upon the exploitation of the genetic tractability of unicellular organisms to identify the endogenous molecular mediators of death. This, in turn, would allow the construction of testable models for apoptotic death in higher cells.

In light of the above, we have explored the possibility that the yeast *S. pombe* may be a model system for certain aspects of apoptotic death. In this report we have sought to determine whether the pro-apoptotic proteins bak or bax elicit a selectable phenotype upon expression in *S. pombe*. Such a phenotype need not, in our opinion, form the basis of a demonstration of classical apoptotic morphology but should establish the basis for a genetic analysis designed

to identify the components of the bak/bax controlled pathway(s) in yeast. In this report we demonstrate that bak expression in *S. pombe* rapidly commits cells to a cell cycle specific lethality which is rescued by co-expression of bcl-2 and bcl-x_L. In addition, we identify an *S. pombe* protein which interacts with bak upon two-hybrid analysis and demonstrate that this interaction is necessary for bak induced lethality in *S. pombe*.

Results

Bak expression in *S. pombe* is lethal

To evaluate the phenotype in *S. pombe* upon expression of the pro-apoptotic proteins bak and bax, cDNAs were subcloned into the REP_5 multi-copy plasmid under the control of the thiamine repressible *nmt1* promoter (Maundrell 1990; Maundrell 1993) and transformed into a host *S. pombe* haploid strain (Materials and Methods). To asses cell division upon expression, growth was tested on solid MM lacking thiamine (Figure 1a). In the bax expressing strain growth was significantly slower than the control, while upon bak expression no growth was observed.

A time course of cell density was performed in order to gain a more detailed understanding of cell behaviour upon bak or bax expression (Figure 1b). After 20 h of logarithmic growth in MM lacking thiamine (2 h after maximal mRNA expression was achieved see Figure 1c), the growth rate of cultures expressing bak or bax decreased. This difference corresponds to a rate of growth of one division every 9 h compared to the wild-type rate of one division every three and a half hours. After 60 h, growth of the bak expressing culture had essentially ceased while the bax expressing culture, which exhibits the same time-course of mRNA induction as the bak culture (data not shown), continued to grow at the compromised rate.

To determine whether bak expression results in reversible cell cycle arrest or lethality, the extent to which the phenotype upon bak expression could be reversed by inhibiting the *nmt1* promoter with thiamine, was assessed (Figure 2). After 14 h of growth in the absence of thiamine, at which time bak mRNA is first detected (Figure 1c), cells first began to commit to death and after 18 h 100% of the cells became committed to die. Since the *nmt1* promoter is fully repressed by thiamine within 3 h (Maundrell 1990) and since the assessment of death is made 14 days after the addition of thiamine to the cells, we conclude that bak is lethal in *S. pombe* and that once bak expression is initiated, cells rapidly pass a critical point beyond which commitment to death is irreversible.

Bak lethality is rescued by co-expression of bcl-2, bcl-x_L and E1B 19K

Protection from the bax induced slow growth and bak lethality described above is provided by co-expression of bcl-2 or bcl-x_L, while only bak induced lethality is rescued by co-expression of the adenovirus E1B 19K protein (data not shown). The most simple explanation for these results is that protection from lethality is a consequence of heterodimer formation between bcl-2 or bcl-x_L and bak or bax leading to inactivation. The apparent specificity of E1B 19K rescue most likely reflects a difference in affinity for bak and bax.

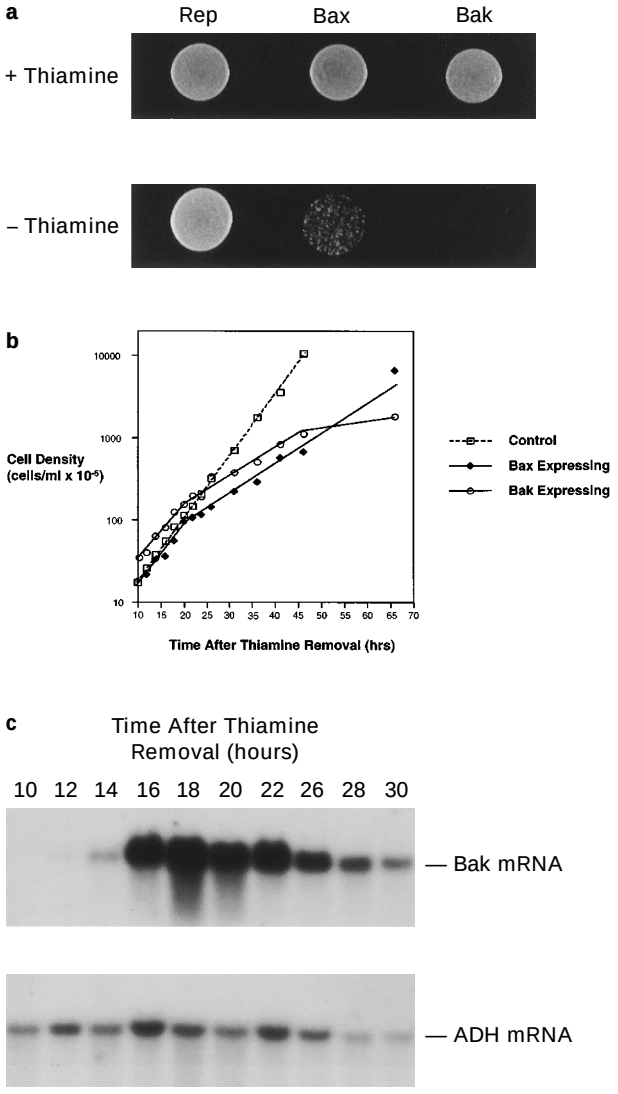


Figure 1 Effect of bak or bax expression upon growth rate in *S. pombe*. (**a**) *S. pombe* transformed with REP_5, REP_5 : bax or REP_5 : bak was grown for 20 h in liquid MM either lacking thiamine to induce expression or containing 4 μM thiamine for controls. Cell concentrations were estimated with a haemocytometer and a volume of 20 μl, containing 100 000 cells, was deposited onto MM plates either with 4 μM thiamine (in the case of controls) or without thiamine. Photographs were taken after 48 h of growth at 30°C. (**b**) *S. pombe* transformed with REP_5 (- - -□- - -), REP_5 : bax (—◆—) or REP_5 : bak (—○—)was grown to logarithmic phase in MM containing 4 μM thiamine. At the zero time point cells were washed three times with MM lacking thiamine and grown in the same media at 30°C. To maintain the cells in logarithmic growth phase, cultures were regularly diluted and each dilution was taken into account for graphic representation of cell density on the y axis so that the slope of the line is representative of rate of growth. (**c**) Time points for Northern analysis were taken from an experiment performed as described for Figure 1b. At the time points indicated, total RNA was isolated from 10^8 cells and Northern blot analysis was performed as described in Materials and Methods. The same nylon filter was probed with bak cDNA and, as a control for sample loading, alcohol dehydrogenase (ADH) cDNA.

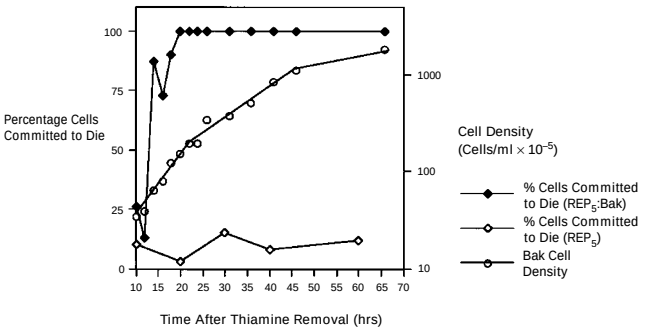


Figure 2 Bak expression rapidly commits *S. pombe* cells to death. *S. pombe* cells transformed with REP_5 : bak or REP_5 (as control) were grown in liquid MM lacking thiamine. At different time points after thiamine removal from the culture media, cells were removed from the culture and spread onto solid MM containing 4 μM thiamine. After 14 days at 30°C the percentage cells committed to die was defined as the number of colonies which did not grow relative to the total number of cells spread. The growth curve of bak expressing cells from Figure 1b is shown to ease comparison.

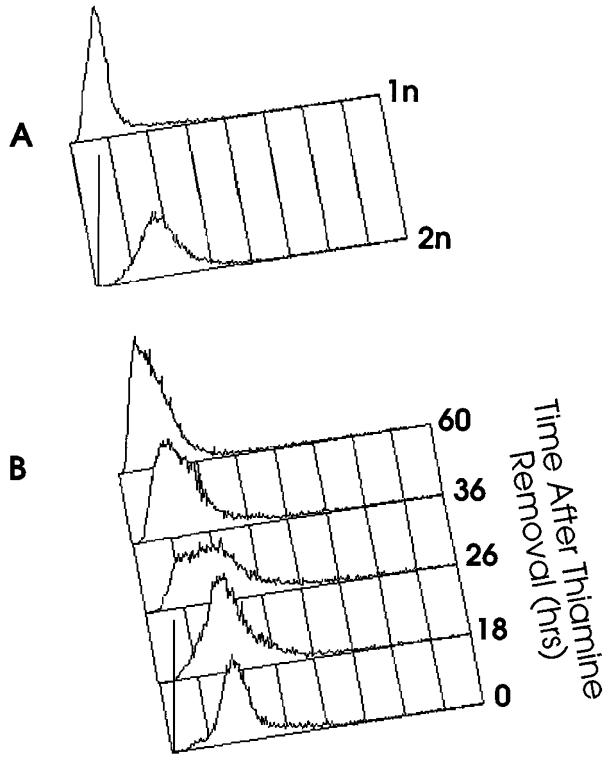


Figure 3 Bak expression leads to an accumulation of cells with a 1n DNA content. (**a**) As a 1n DNA content control, *S. pombe* was grown to stationary phase in nitrogen-limited medium (NLM) and flow cytometry analysis performed. The 2n DNA content control was a wild-type logarithmically growing *S. pombe* culture. (**b**) *S. pombe* transformed with REP_5 : bak was grown in MM lacking thiamine to induce bak expression and the DNA content of the cells at the times shown after thiamine removal measured by flow cytometry analysis. In both graphs shown, the x-axis represents fluorescence as a measure of DNA content and the third dimension axis is representative of cell number.

Heterodimer formation has been demonstrated for all these interactions by *in vivo* and *in vitro* experiments (Farrow and Brown 1996), although it is possible that rescue is due to competing pathways in yeast influenced by bcl-2, bcl-x_L or E1B 19K expression. It should be noted that while the expression of bak or bax elicits observable phenotypes in *S. pombe* the expression of the bcl-2 family members which have apoptotic protective functions in higher cells, such as bcl-2 or bcl-x_L, elicit no detectable growth effect when expressed alone.

Bak expression in *S. pombe* provokes a cell cycle specific lethality

To gain an understanding of the nature of the lethality caused by bak expression, an analysis of the cellular DNA content and morphology upon expression was performed (Materials and Methods). DNA content was measured by flow cytometry analysis on REP_5 : bak transformed cells taken at different time points after thiamine removal from the culture media (Figure 3). When maximal bak mRNA expression was achieved (18 h, see Figure 1c) all the cells in the population had the 2n DNA content characteristic of logarithmically growing *S. pombe*. By 26 h, cells with a 1n DNA content began to appear and the proportion of 1n cells increased with time until the culture had ceased dividing. By 60 h, 83% of cells in the culture had a 1n DNA content. It should be noted that the DNA content of control cells was not affected by long term growth in the absence of thiamine. These results suggest that bak expression in exponentially growing *S. pombe* hinders progression into S-phase of the cell cycle so that the terminal phenotype of the majority of cells is in G1.

Microscopic analysis of cells stained with Hoechst (nuclear stain) and Calcofluor (septal stain) (Materials and Methods) demonstrated that, although all of the bak expressing cells are irreversibly committed to die (see Figure 2), approximately 90% of expressing cells had a morphology similar to that which is found in wild-type cultures except for a slightly larger length and diameter. Cells with a distinctive morphology not found in wild-type cultures however, began to appear 20 h after thiamine removal from the culture (Figure 4). Cells with an asymmetric nuclear distribution across the axis of the septum are observed upon bak expression (Figure 4b, c, d and h) and these cells are capable of cytokinesis (Figure 4d) giving rise to anucleate cells (Figure 4e). Cells with brightly staining septa (Figure 4b, c, f, g and h), multiple septa (Figure 4f and g), septa which deviate from the perpendicular axis of the cell (Figure 4b and h) and abnormally elongated cells (Figure 4i) are also observed upon bak expression. The proportion of these phenotypes, found in a bak expressing culture is shown in Table 1.

In order to determine DNA content of the aberrant cells shown in Figure 4, cells expressing bak after 36 h growth in the absence of thiamine (1 : 1 ratio of 1n : 2n see Figure 3) were separated by FACS into a 1n and 2n population. Microscopic analysis of these two populations stained with Hoechst and Calcofluor revealed that the phenotypes shown in Figure 4 were found only in the 2n population.

These cells appear therefore to have overcome the G1 cell cycle block imposed by bak expression, completed S-phase and failed to complete M-phase. They remain either in the G2 growth phase of the cell cycle giving rise to abnormally long cells, or begin to mis-coordinate the timing of cell-cycle events with septation and cytokinesis

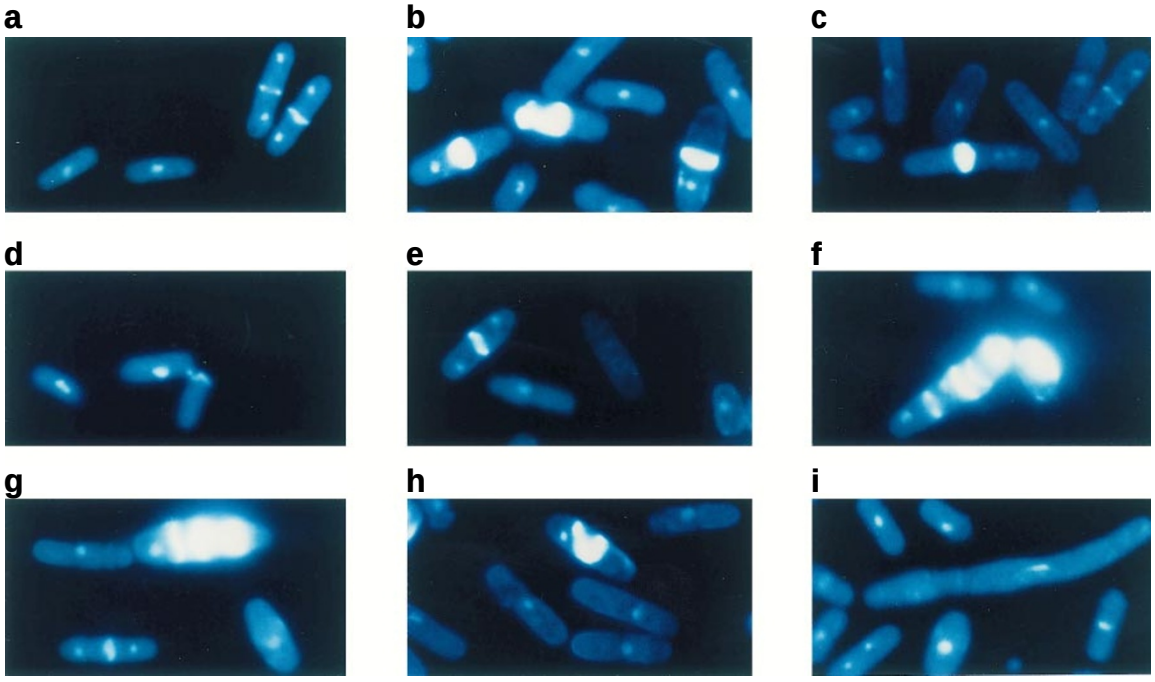
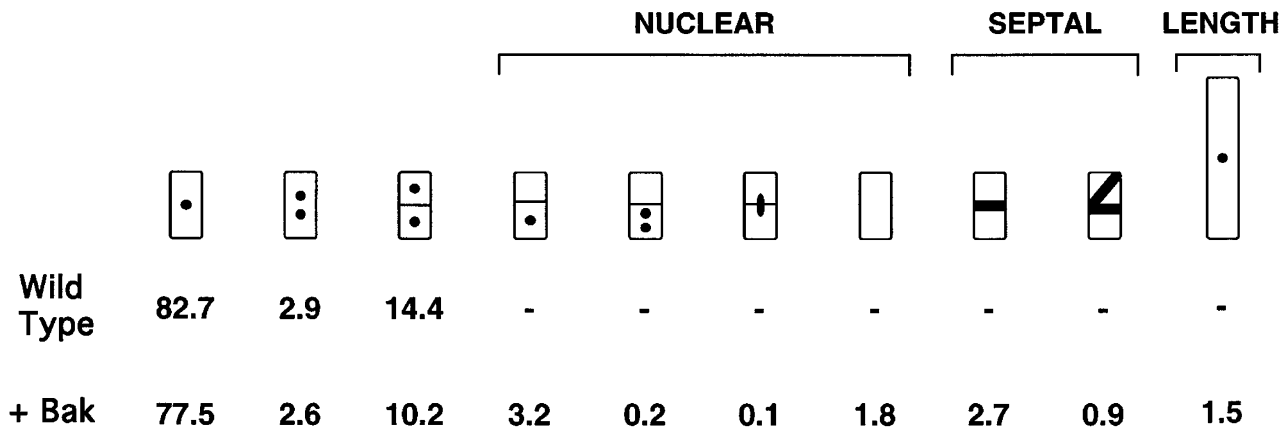


Figure 4 Expression of bak in *S. pombe* provokes a characteristic phenotype. *S. pombe* transformed with REP_5:bak was grown in MM lacking thiamine for 42 h and stained with Hoechst (nuclear stain) and Calcofluor (septal stain). Wild-type cells (**a**) are shown for comparison with bak expressing cells (**b** to **i**). Cells with an assymetric nuclear distribution across the septum are shown (**b**, **c**, **d** and **h**). Cells with an intense septal staining (**b**, **c**, **f**, **g**, **h**), septum deviated from the perpendicular axis of the cell (**b** and **h**) and multiple septated cells (**f** and **g**) are shown. Also shown is an abnormally elongated cell (**i**) a cytokinesing cell which has an assymetric nuclear distribution across the septum (**d**) and an anucleate cell (**e**). All photographs were taken with a magnification of 1000×.

Table 1 Percentages of phenotypes upon bak expression



Wild-type *S. pombe* and *S. pombe* transformed with REP_5:bak were maintained at logarithmic growth phase in MM lacking thiamine for 60 h and stained with Hoechst and Calcofluor. For both experimental subjects a total of 3000 cells were counted and orgainsed into the phenotypes diagrammatically shown. It should be noted that for simplicity, in each abnormal phenotypic category (i.e. abnormal nucleus, septum or length) only one single characteristic is expressed as a percentage and represented in each cellular diagram. As a consequence there is overlap between the phenotypes and for the cells transformed with REP_5:bak the total percentage is greater than 100%

occurring before M-phase completion and repeated septum deposition occurring, sometimes in an incorrect orientation.

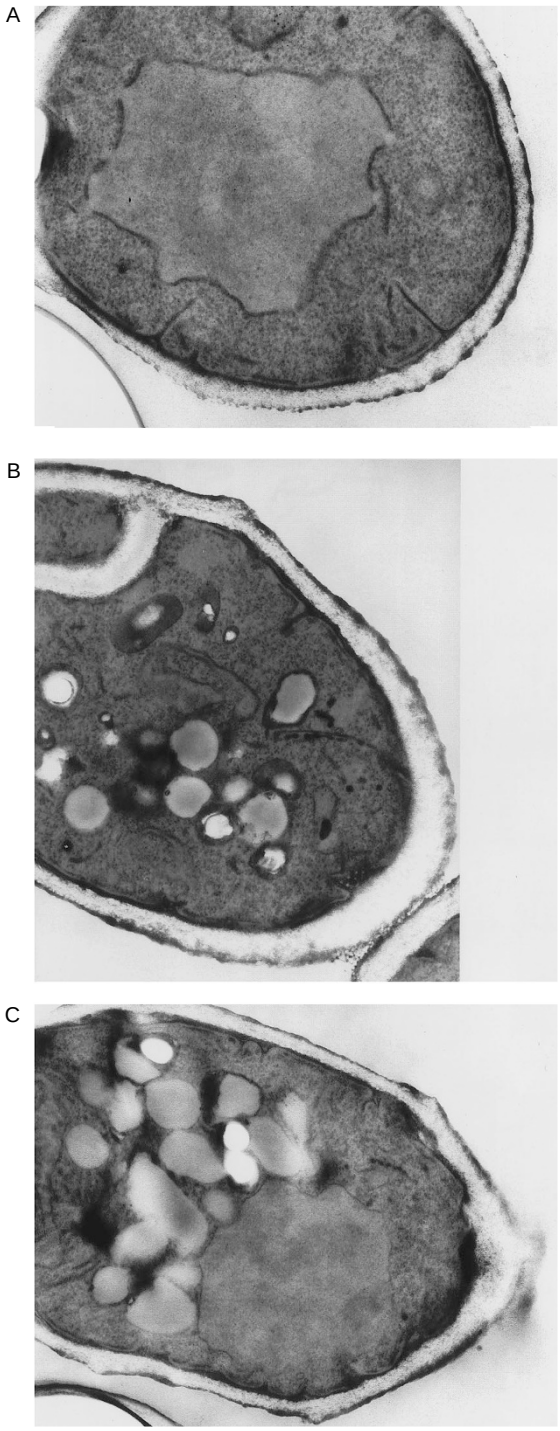


Figure 5 Bak expression in *S. pombe* results in the accumulation of large vesicular structures. *S. pombe* cells transformed with REP_5 as control (**a**) or REP_5 : bak (**b** and **c**) were grown for 42 h in liquid MM lacking thiamine. Cells were then fixed and treated as described in Materials and Methods.

Similar analysis of bax expressing cells demonstrated the presence, in comparable proportions, of all the aberrant phenotypes described for bak. In addition, upon bax expression the percentage of cells with a 1n DNA content was elevated relative to the control (approximately 20% compared to 8% for control – results not shown) but 1n cells did not accumulate with prolonged expression.

In order to examine more closely the morphology of bak expressing cells in comparison with wild type *S. pombe* both cultures were analyzed by electron microscopy. The most striking change upon bak expression was the appearance, in all the expressing cells analyzed, of large vesicular structures (Figure 5b and c). In the majority of bak expressing cells the nuclei appeared to have normal morphology. After a long period of bak expression in *S. pombe* (72 h) genomic DNA degradation can be observed on agarose gels but this is unlikely to be a primary determining factor in the bak-induced cell death, since commitment to death occurs very rapidly upon induction of bak expression (See Figure 2).

Bak lethality is mediated in part through interaction with Cnx1

In order to determine whether the bak induced lethality was dependant on the physical interaction of bak with an *S. pombe* protein and to isolate this protein, an *S. pombe* two-hybrid cDNA library (Clontech) was screened with human bak protein fused to GAL-4 as 'bait'. Ten million cDNA clones were screened and 31 positives were isolated that interacted with the bak protein and not with a range of non-specific baits. These were extracted and sequenced. All 31 positives were GAL4 fusions with the *S. pombe* cDNA encoding the calnexin homologue Cnx1 (Figure 6a) which is an ER integral membrane protein with a cytosolically directed C-terminus (Parlati *et al*, 1995). Calnexin is an ER protein expressed in all eukaryotic cells which functions as a molecular chaperone and as a component of the ER quality control apparatus (Bergeron *et al*, 1994). This positive was of particular interest as bak and other bcl-2 family members are known to co-localise (in higher cells) with calnexin in the ER (Chen-Levy *et al*, 1989; Jacobson *et al*, 1993 and R. Brown unpublished). The smallest clone isolated contained only the C-terminal 99 amino acids of Cnx1, sufficient to code for the transmembrane and C-terminal tail of the protein. No other members of the bcl-2 family, with the exception of bax, demonstrated a positive interaction with Cnx1 when tested by two-hybrid analysis. In the case of bax the interaction was significantly weaker than that seen with bak, being only just above the background for detection. This difference in affinity for Cnx1 may underlie why bak and bax appear to elicit the same phenotype with different severities.

In order to test whether interaction between the cytosolic domain of Cnx1 and bak is responsible for lethality in *S. pombe* a strain deleted for wild type *cnx1* was utilised. The normally lethal effect of *cnx1* deletion was averted by expression of either the wild-type *cnx1* gene or a C-terminal truncated version which terminates at amino acid 524. This truncated Cnx1 still inserts into the ER membrane and complements deletion of the wild-type gene fully (Parlati *et*

al, 1995) but does not contain the cytosolic domain which binds bak in two-hybrid experiments. While both the strains utilised were equally viable, the presence or absence of the cytosolic domain of Cnx1 altered the phenotype observed upon bak expression. Bak was lethal in the strain expressing wild-type Cnx1 but not in the strain expressing C-terminal truncated Cnx1 (Figure 6b). Although deletion of the cytosolic domain of Cnx1 was sufficient to allow cells to overcome bak induced lethality it can be seen from Figure 6b that growth is still slower than controls. It is not clear whether this compromised growth rate is representative of the doubling time of the cells or whether it is representative of a Gaussian distribution of severity of phenotype in the population.

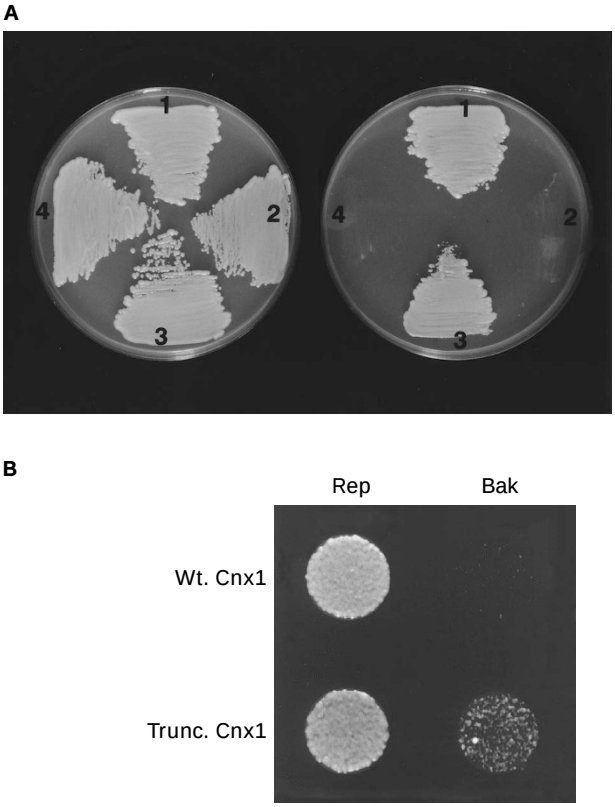


Figure 6 Bak interaction with Cnx1 is necessary for lethality in *S. pombe*. (**a**) In order to demonstrate the two hybrid interaction between bak and Cnx1 the *S. cerevisiae* strain Y190 was transformed with a plasmid containing the full length bak cDNA fused to the binding domain of the yeast GAL4 protein. This strain was then transformed with a vector containing the yeast GAL4 DNA-binding domain fused to either the *cnx1*, bcl-x or bax cDNAs. These strains were then streaked onto plates containing either selective medium alone (left plate) or selective medium with the addition of 25 mM 3-aminotriazole (3-AT), right plate. A GAL4-dependant trans-activation of the HIS gene promoter in these strains permits growth in the presence of 3-AT and demonstrates a positive interaction. Both plates are divided into 4 sectors. Sector 1=bak vs. cnx1, Sector 2=bak vs. GAL4 activation domain (negative control), Sector 3=bak vs. bcl-x (positive control), Sector 4=bak vs. bax (negative control). Interaction of bak with Cnx1 and bcl-x in this two hybrid experiment also resulted in strong lac-z staining confirming the interaction with the second assayable marker (data not shown). (**b**) The cells shown are deleted for the *S. pombe cnx1* gene function and viability is maintained by expression of either wild type Cnx1 or a truncated Cnx1 which terminates at amino acid 524. These two strains were transformed with REP_5 or REP_5 : bak and grown for 20 h in liquid MM lacking thiamine to induce expression. Cell concentrations were estimated with a haemocytometer and a volume of 20 μl, containing 50 000 cells was deposited onto an MM plate lacking thiamine. The photograph was taken after 48 h of growth at 30°C.

Interaction between bak and Cnx1 results in a dominant lethal effect

A simple model to explain why the association of bak and Cnx1 contributes to lethality in *S. pombe* is that the interaction blocks normal Cnx1 function. If this were the case, bak induced lethality in a wild-type *S. pombe* genetic background should be rescued by the overexpression of either wild-type or C-terminally truncated forms of the Cnx-1 protein. No rescue from bak lethality in *S. pombe* was observed upon over-expression of either of these two forms of calnexin (data not shown). While the failure of full length Cnx1 to rescue bak lethality might be explained by insufficient levels of Cnx1 protein, this cannot be the case for the C-terminally truncated Cnx1 since it does not contain the bak binding domain but does fully complement the lethal *cnx1* gene deletion (Parlati *et al*, 1995). The failure to rescue bak lethality, in a wild-type *cnx1* background, by overexpression of two different forms of Cnx1 suggests that the interaction between bak and Cnx1 results in a dominant lethal effect perhaps due to the propagation of a lethal signal or the recruitment of additional interactors to the bak Cnx1 complex.

Discussion

Overexpression of the killing Bcl-2 family members bak and bax has been shown to be lethal across a diverse selection of evolutionarily distinct cell types ranging from humans, *C. elegans* and now the yeast *S. pombe*. In addition, across this same evolutionary distance the protecting family members bcl-2 and bcl-x_L have been shown to protect from that lethality. Bak expressing *S. pombe* cells have a terminal phenotype in which the majority of cells are arrested in the G1 phase of the cell cycle while the remainder of cells, unable to complete M-phase, mis-coordinate the timing of subsequent events in the cell cycle such as septation and cytokinesis. In bax expressing *S. pombe*, cells enter S-phase less efficiently than wild type and also mis-coordinate post M-phase events. Bak and bax appear to differ therefore only in the severity of phenotype upon expression, thus suggesting that bak and bax have at least one common mode of action in *S. pombe*.

Electron microscopy analysis of cells expressing bak reveals a dramatic accumulation of large vesicular structures. Intriguingly, similar morphological changes have been observed in *Dictyostelium* cells stimulated, with physiological inducers, to undergo a programmed cell death (Cornillon *et al*, 1994), in *C. elegans* cell death (Robertson and Thomson 1982) and in some instances of higher eukaryote cell death (Clarke 1990). It remains to be seen whether this cellular feature is a consequence of a common molecular mechanism of death.

A two-hybrid screen designed to identify *S. pombe* proteins which interact with bak, isolated the *S. pombe* calnexin homologue *cnx1*. Genetic analysis demonstrates that the Cnx1 domain which binds to bak in two-hybrid experiments, is necessary for bak lethality in *S. pombe*. These results strongly suggest that bak lethality in *S.*

pombe is, at least in part, caused by the interaction between bak and the cytosolic domain of Cnx1. Since bak expression in a genetic background deleted for the Cnx1 cytosolic domain still results in a compromised growth rate, it is likely that the lethal phenotype in *S. pombe* is driven by the interaction of bak with multiple proteins and that only one of those lethal interactions is with Cnx1. Interestingly, bax also interacts with Cnx1 in two-hybrid experiments but with significantly less affinity than bak. This difference in affinity for Cnx1 perhaps underlies why bak and bax appear to elicit the same phenotype with different severities.

Attempts to protect from bak lethality in *S. pombe* by co-expression of full length and C-terminally truncated Cnx1 were unsuccessfull thus suggesting that bak lethality is not due to a recessive loss of Cnx1 function upon bak binding. Support for this is further provided by the observation that the phenotype associated with *cnx1* gene disruption in *S. pombe* is not similar to the bak expression phenotype described in this report (E. Meldrum unpublished). It therefore appears that the bak Cnx1 interaction provokes a dominant lethal effect perhaps due to the propogation of a lethal signal or the recruitment of additional interactors to the bak Cnx1 complex. The nature of this dominant lethality is currently under investigation by classical genetic approaches to generate bak resistant strains, by *cnx1* mutagenesis and by biochemical analysis of Cnx1 function in bak expressing cells.

In our opinion, the significance of bak lethality in *S. pombe* would be emphasised if it was possible to abrogate the same cellular functions by expressing another pro-apoptotic protein, not in the bcl-2 family but on the same programmed death pathway. This has been tested with the *C. elegans* pro-apoptotic protein Ced-4. Expression of Ced-4 in *S. pombe* induces a lethality qualitatively similar to that described in this report i.e. accumulation of 1n cells (50% after 50 h growth in the absence of thiamine) and mis-timing of post M-phase events in the cell cycle to give rise to phenotypes shown in Figure 4. The nature of this Ced-4 lethality suggests that, even though they possess no sequence similarity, bak and Ced-4 interact with the same cellular function in *S. pombe*. The mechanisms of Ced-4 lethality in *S. pombe* are currently under investigation.

When one considers the evolutionary distance across which killing members of the bcl-2 family have now been shown to be lethal one has to propose that, unless bak is killing in these diverse cell types by chance alone, bak is interacting with a highly conserved cellular target or function. While yeast does not appear to possess an apoptotic programme, it cannot be discounted that one or more pathways in yeast may have been adapted by higher eukaryotes to provide an apoptotic pathway or alternatively, that bak may abrogate the same highly conserved cellular function in both yeast and higher eukaryotes. It is consistent with this reasoning to report that bak expression in *S. pombe* interferes with normal cell cycle progression, as components which regulate cell cycle progression are highly conserved across evolution (Morgan 1995). In addition, it is demonstrated that bak induced lethality in *S. pombe* is achieved in part through the interaction between bak and the ER localised calnexin homologue Cnx1. There is 50% sequence homology and a large degree of functional conservation between the human and *S. pombe* calnexin proteins. Bak in *S. pombe* appears therefore to be interacting with highly conserved cellular functions thus suggesting that the same bak lethal targets may be conserved between *S. pombe* and higher cells.

Bcl-2 family members in higher cells are known to be localised in the mitochondria, nuclear envelope and co-localised with calnexin at the ER (Gonzalez-Garcia *et al*, 1994; Krajewski *et al*, 1993; Chen-Levy *et al*, 1989; Jacobson *et al*, 1993), and it appears that the precise subcellular localisation of their function is cell type dependant (Zhu *et al*, 1996). It remains to be seen whether the highly conserved calnexin function also interacts with bak protein in higher cells. If so then this interaction may uncover part of the mechanism by which bak, resident in the ER (R. Brown unpublished), elicits its lethality. This analysis in higher cells, and the identification of the additional mechanisms by which bak provokes lethality in *S. pombe*, will be of central importance in the final assessment of bak mediated lethality in *S. pombe* as a model system for studying bcl-2 family member function.

The data described in this report establish the basis for genetic screening for the targets of bak/bax lethality in *S. pombe*. The observation that the *S. pombe* calnexin homologue Cnx1 is one of the lethal partners of bak, represents the first identification of a lethal partner of bak in any system. Further genetic analysis of the additional mechanisms of bak lethality in *S. pombe* will permit the identification of all the targets of bak action in *S. pombe*, and thus uncover additional functional principles for bcl-2 family members which can be tested in higher eukaryote cells.

Materials and Methods

Strains and culture media

All experiments described utilised the haploid *S. pombe* strain Sp30 (h^--leu1-32 ura4-D18 ade6-704 his5-303) based on the original wild-type 975h^+ strain from the Berne collection. All strain constructions were performed by standard genetic procedures. All experiments were carried out with cells in logarithmic growth phase ($<1\times10^7$ cells/ml). Minimal medium (MM) used for liquid cultures or for plates had the same composition as described (Moreno *et al*, 1991), except that the NH_4Cl concentration was at 2.5 g/litre instead of 5 g/litre and the appropriate amino-acids were omitted in order to ensure plasmid maintenance. Nitrogen limiting media (NLM) had the same composition as MM, except that the NH_4Cl concentration was at 267 mg/litre. To induce the *nmt1* promoter, cells grown in liquid MM containing 4 μM thiamine were washed three times with 10 ml of MM lacking thiamine and cultured in this medium. In all experiments, cell densities were determined with a haemocytometer.

Northern analysis

Total mRNA was isolated as previously described (Elder *et al*, 1983) and separated electrophoretically on a 1% agarose gel containing 2.2 M formaldehyde (Sambrook *et al*, 1989). RNA was transferred to N-Hybond nylon filters, and hybridised for 12 h with a ^{32}P-α-dCTP random labelled probe in 50% formamide buffer at 42°C. Three

washes in 1 × SSC, 0.1% SDS (w/v) at room temperature and a single wash with 0.1 × SSC, 0.1% SDS (w/v) for 90 min at 60°C were performed and blots were exposed for 44 h at −70°C with an intensifying screen.

FACS analysis

For each time point, 1×10^7 cells were collected by centrifugation at 1000 × *g* for 5 min, washed three times in PEM (100 mM PIPES (sodium salt), 1 mM EGTA, 1 mM $MgSO_4$) and resuspended in 200 μl of PEM. Half of this cell suspension was Hoechst stained by the addition of 2 μl of a 6 mg/ml frozen stock solution followed by a 30 min incubation at 37°C. Cells were then fixed in 4 ml of 2% (v/v) Paraformaldehyde and stored in the dark at 4°C. Flow cytometry was performed with a FACStar plus (Becton Dickinson, Erembodegem, Belgium) equipped with a UV argon ion laser (Spectra Physics, Les Ullis, France) emitting 120 mW at 351–364 nm. The blue fluorescence of Hoechst 33342 was measured through a 450/10 band pass filter. 10 000 cells were recorded for each time point and Hoechst fluorescence was displayed on a linear scale with the pulse area measured using the pulse processing module.

UV microscopy

5×10^7 cells were centrifuged at 1000 × g for 5 min. Cell pellets were washed twice with PEM (see above) and resuspended in 200 μl of PEM containing Calcofluor. The Calcofluor solution was prepared immediately before use by dissolving 5 mg of Calcofluor in 1 ml of PEM. Undissolved material was removed by centrifugation at 10 000 × g for 5 min. The cells were resuspended in 200 μl of a fivefold dilution of the Calcofluor supernatant prepared above, then incubated on a rotary inverter in the dark at room temperature for 15 min. Cells were washed three times with, and finally resuspended in, 1 ml of PEM. For nuclear staining, 2 μl of a 6 mg/ml frozen stock solution of Hoechst was added to 100 μl of cells stained with Calcofluor as described above. Cells were incubated for 30 min at 37°C. Cells were mixed with an equal volume of antifade (Molecular probes) and after vortexing, 3 μl was applied to a microscope slide, a coverslip applied and sealed with nail polish. Cells were viewed with a fluorescence microscope (Zeiss) using a UV filter (487702 A).

Electron microscopy

S. pombe cells transformed with REP_5 or REP_5 : bak were grown for 42 h in liquid MM lacking thiamine. Cells were then fixed with Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate) for 15 min at 20°C followed by post-fixation in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h at 5°C. Cells were then pelleted, dehydrated and embedded in Epon. Sections were cut on a Riechert-Jung Ultracut E microtome and stained in aqueous uranyl acetate and lead citrate. Sections were examined in a Phillips CM12 transmission electron microscope.

Acknowledgements

We thank Francesco Parlati and David Thomas for the *S. pombe cnx1* deletion strain and *cnx1* expression plasmids and the MRC Laboratory for Molecular Cell Biology for assistance with electron microscopy. The authors would also like to thank Jonathan Knowles for his vision, leadership and support and Stuart Farrow, Jean-Claude Martinou, Kinsey Maundrell, Mark Payton, Roberto Solari and Jurg Tschopp for discussions, support and review of the manuscript.

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