S. pombe Pbh1p: an inhibitor of apoptosis domain containing protein is essential for chromosome segregation

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Received 20 September 1999

Abstract Proteins containing the baculovirus inhibitor of apoptosis repeats (BIR domains) have been identified in a wide range of species. BIR domain containing proteins are thought to inhibit caspases and thereby cause inhibition of apoptosis. A BIR domain containing protein has been recently identified by the *Schizosaccharomyces pombe* genome sequencing project. However, caspase-like proteins have not been found in yeasts, suggesting that the BIR domain containing proteins might play a fundamental role in cell regulation, in addition to their well-characterized role in inhibition of apoptosis. In this study, we have characterized Pbh1p, an *S. pombe* BIR domain containing protein. Construction and analysis of a null mutant in *pbh1*$^+$ revealed that *pbh1*$^+$ is essential for cell viability. Moreover, cells devoid of Pbh1p are defective in chromosome condensation and chromosome segregation. Thus, proper chromosome segregation requires the function of Pbh1p. Over-production of Pbh1p led to abnormalities in mitosis and cytokinesis, suggesting that the levels of Pbh1p are important for regulation of mitosis and cytokinesis.

Key words: BIR domain; Apoptosis; *pbh1*; Mitosis; Fission yeast

1. Introduction

Inhibitor of apoptosis proteins (IAPs) are known to be one of the major components in the highly conserved apoptotic machinery in metazoans [1]. BIR (*B*aculovirus *I*AP *r*epeat) domains, which were initially identified as essential for the anti-apoptotic activity of the IAPs [2,3], are now known to be conserved in a wide range of proteins that are both structurally and functionally diverse. The IAP homolog in *Caenorhabditis elegans*, BIR-1, plays a role in embryonic cytokinesis [4] and survivin, a human BIRp, is cell cycle regulated and associates with the mitotic spindle [5]. Identification of BIR domain containing proteins in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, unicellular organisms that have no known apoptosis mechanisms, clearly indicates that BIR domains are not specific for apoptosis but instead might have a more conserved and basic cellular function.

The fission yeast, *S. pombe*, has proven to be an excellent model organism to study cell cycle mechanisms, including those regulating mitosis and cytokinesis. For example, cell cycle regulatory molecules identified from studies in *S. pombe* are known to be highly conserved across a wide range of phyla [6–8]. The identification of a BIR domain containing protein in *S. pombe*, Pbh1p (*p*ombe *b*ir *h*omolog), prompted us to investigate its possible role(s) in cell cycle regulation. In this paper, we describe the construction and analysis of the *pbh1* null mutant and also investigate the role of this protein in regulating the cell cycle.

2. Materials and methods

2.1. Strains and media

S. pombe cells were grown in yeast extract (YES) medium or minimal medium (EMM2) with appropriate supplements [9]. For disruption of *pbh1*$^+$, a diploid strain was made by crossing haploid strains MBY101(*h*$^-$, *ade6-210, ura4D-18, leu1-32*) and MBY104 (*h*$^+$, *ade6-216, ura4-D18, leu1-32*). An *S. pombe* haploid wild-type strain MBY192 (*h*$^-$, *ura4-D18, leu1-32*) was used for the overexpression studies.

2.2. *pbh1*$^+$ gene disruption

For *pbh1*$^+$ gene disruption, the entire 3 kb coding region of *pbh1*$^+$ was replaced with the 1.8 kb *S. pombe ura4*$^+$ gene. A linearized DNA molecule consisting of the *pbh1::ura4*$^+$ allele, retaining approximately 1 kb of flanking sequence to provide homology on both sides, was transformed into the wild-type diploid, MBY101/104, for integration into the *pbh1*$^+$ locus by homologous recombination. A diploid strain, MBY527, carrying a replacement of one of the copies of *pbh1*$^+$ by the *pbh1::ura4*$^+$ allele, was identified by screening uracil prototrophic colonies by polymerase chain reaction. MBY527 was sporulated and tetrads were dissected on rich media. For phenotypic characterization of the *pbh1::ura4*$^+$ allele, MBY527 was sporulated on EMM plates lacking uracil and adenine and the spores were subjected to treatment with glusulase in order to digest the wall of the asci. Spores obtained from MBY527 were germinated in EMM lacking uracil to only allow germination of *pbh1::ura4*$^+$ spores.

2.3. Plasmids

Plasmid REP3X [10] with the thiamine-repressible *nmt* promoter was used for overproduction of Pbh1p. For this purpose, primers MOH325 (5′-GCATGTGTCGACATGAAACCGATAACGTCTTC-3′) and MOH375 (5′-GCATGTAGATCTTTATAAGTTTTGTATG-TATTCTTCCGC-3′) were designed to introduce a *Sal*I site at the ATG and a *Bgl*II site downstream of the stop codon. Primers MOH374 (5′-AATGGCAGTTTGGCTGTCC-3′) and MOH327 (5′-GCCATCAACTTCATCTCCCGATATGAC-3′), which contained an internal *Hin*dIII site, were used in combination with MOH325 and MOH375 respectively, to amplify *pbh1*$^+$ from *S. pombe* genomic DNA. The *pbh1*$^+$ derivative of pREP3X was constructed in a trimolecular ligation involving a 1.7 kb *Sal*I-*Hin*dIII fragment, a 1.5 kb *Hin*dIII-*Bgl*II fragment and pREP3X digested with *Sal*I and *Bam*HI.

2.4. Fluorescence microscopic methods

Cells were fixed with 3.7% formaldehyde for staining with 4′,6-diamidino-2-phenylindole (DAPI) or with methanol for staining with tubulin antibodies (TAT1, a generous gift from Dr. Keith Gull) at a dilution of 1:25. Secondary antibodies used were the Alexa 594-conjugated anti-mouse IgG at a dilution of 1:100. Methods used for immunofluorescence microscopy were essentially as described before [11].

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0014-5793/99/$20.00
PII: S0014-5793(99)01329-0

3. Results

3.1. Identification of a BIR domain containing protein in S. pombe

An *S. pombe* predicted open reading frame (ORF) with amino acid sequence similarity to the inhibitor of apoptosis family of proteins was identified by the *S. pombe* genome sequencing project. The gene encoding this ORF, *pbh1*$^+$ (which we designated *p*ombe *b*ir *h*omolog 1), resides on cosmid 962 on *S. pombe* chromosome III. IAP family proteins are characterized by the presence of a ~70 amino acid domain referred to as the 'BIR domain', originally found in the baculovirus inhibitor of apoptosis proteins [4]. The predicted amino acid sequence of *S. pombe* Pbh1p was used as a query sequence to search available databases. Pbh1p was most closely related to *Mus musculus* IAP-related protein MIAP-2 and was also related to IAP-like proteins from human, chicken, *Drosophila*, and *C. elegans* (Fig. 1). Inspection of the amino acid sequence of Pbh1p revealed the presence of two imperfectly conserved BIR domains, from amino acid 25 to 104 and from amino acid 122 to 199. Amino acid sequence analysis of Pbh1p also revealed the presence of seven potential Cdc2p phosphorylation sites.

3.2. pbh1$^+$ is essential for cell viability

To study the effect of complete loss of function of *pbh1*$^+$ in *S. pombe*, a DNA molecule was constructed in which the coding region of *pbh1*$^+$ was replaced with the selectable marker gene *ura4*$^+$. Linearized DNA containing this *pbh1::ura4*$^+$ allele was used to transform a uracil auxotrophic diploid (MBY101/MBY104). Uracil prototrophs were screened for successful replacement of one of the wild-type copies of *pbh1*$^+$ by the *pbh1::ura4*$^+$ allele by polymerase chain reaction (PCR) and one strain of the genotype *pbh1::ura4*$^+$/*pbh1*$^+$ (MBY527) was identified. MBY527 was sporulated and tetrads were dissected on rich media. Under these conditions, no more than two spores from each tetrad formed colonies (Fig. 2A). Further examination showed that the viable colonies were Ura$^-$, indicating that the *pbh1*$^+$ gene is essential for cell viability. Microscopic examination revealed that the *pbh1::ura4*$^+$ spores had germinated but were arrested as single cells that had performed a catastrophic division.

In order to further characterize the terminal phenotype, *pbh1::ura4*$^+$ spores were germinated and stained with DAPI to visualize the DNA (Fig. 2B). Germinated *pbh1::ura4*$^+$ spores displayed a number of interesting phenotypes similar to that observed in some of the 'cut' mutants. The 'cut' (*c*ell

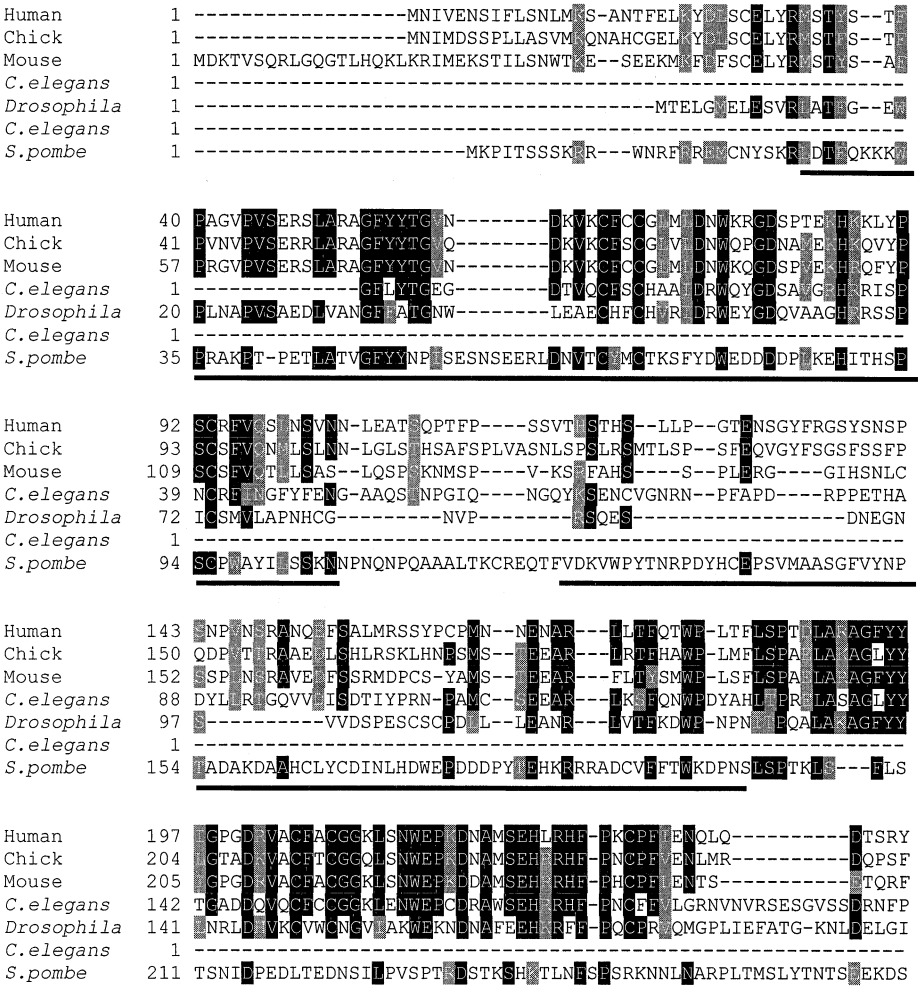

Fig. 1. Alignment of the amino acid sequences of *S. pombe* Pbh1p and the IAPs from mouse (MIAP-2), human (HIAP-1), *Drosophila* (DIAP-2), chicken (IAP-1), *C. elegans* (BIR-1 and BIR-2). Identical amino acids are indicated in black and conservative substitutions are indicated in gray. The two predicted BIR domains in *S. pombe* are underlined.

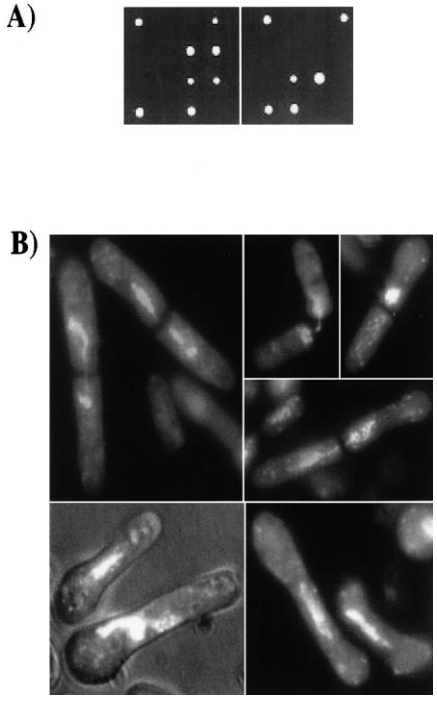

Fig. 2. A: *pbh1*$^+$ is essential for cell viability. Tetrads from *pbh1::ura4*$^+$ cells were dissected and the spores were germinated at 32°C on YES plates. B: *pbh1::ura4*$^+$ cells show a defect in chromosome condensation and segregation. *pbh1::ura4*$^+$ spores were germinated at 30°C in media lacking uracil for 20 h, fixed with formaldehyde and stained with DAPI to visualize DNA.

*u*ntimely *t*orn) mutants have a prominent phenotype of septation and cytokinesis occurring in the absence of chromosome segregation [12]. In some germinated *pbh1::ura4*$^+$ spores, chromosomes were not detected on both sides of the division septum, indicative of failed chromosome segregation. In the vast majority of the germinated spores, chromosomes appeared to be loosely organized and these apparently uncondensed chromosomes were frequently cut by the division septum. In many cases chromosomal DNA was seen to connect the divided cells. No evidence for chromosome condensation was observed upon examining approximately 1000 germinated spores. These data led us to conclude that *pbh1*$^+$ is essential for completion of mitosis and presumably for chromosome condensation.

3.3. Mitotic spindle does not fully elongate in *pbh1::ura4*$^+$ cells

Because of their defect in sister chromatid segregation, we wanted to investigate whether the *pbh1::ura4*$^+$ cells were defective in spindle elongation. To achieve this, germinated *pbh1::ura4*$^+$ spores were fixed and stained with TAT1 antiserum and with DAPI to visualize microtubules and nuclei respectively. Germinated *pbh1::ura4*$^+$spores with interphase nuclei displayed a typical interphase array of microtubules (data not shown). Upon entry into mitosis, *pbh1::ura4*$^+$ spores assembled a short mitotic spindle (cells 1 and 2 in Fig. 3). In some cells, spindles of intermediate length were observed (cell 3 in Fig. 3). However, spindles that span the entire length of the cell, as seen in wild-type cells [13], were never observed. In cells with a division septum, the post-anaphase-like array of microtubules was visualized (cells 4–6, Fig. 3). These observations suggested that failure of proper chromosome condensation and spindle elongation might result in defective chromosome segregation in cells devoid of Pbh1p.

3.4. Overproduction of Pbh1p is toxic and causes chromosome segregation abnormalities

In order to study the effects of overexpression of Pbh1p in wild-type *S. pombe* cells, a DNA molecule was constructed in which the *pbh1*$^+$ coding sequence was fused downstream of the full-strength *nmt1* promoter to produce pREP3X-*pbh1*$^+$. In the presence of thiamine, when the *nmt1* promoter is repressed, the cells carrying pREP3X-*pbh1*$^+$ resembled wild-type cells (data not shown). Interestingly, 20 h after induction of pREP3X-*pbh1*$^+$ expression, by removal of thiamine, approximately 15% of the cells displayed a defect in cytokinesis. These cells accumulated three or four nuclei, due to inefficient cleavage of the division septum (cells 1–3, Fig. 4). In addition, approximately 4% of the cells (from amongst 500 cells observed) showed a defect in chromosome segregation and generated a 'cut' phenotype (Fig. 4, cells 4–7). Such chromosome segregational abnormalities were never observed in cells grown under non-inducing conditions. Whereas cell 4 in Fig.

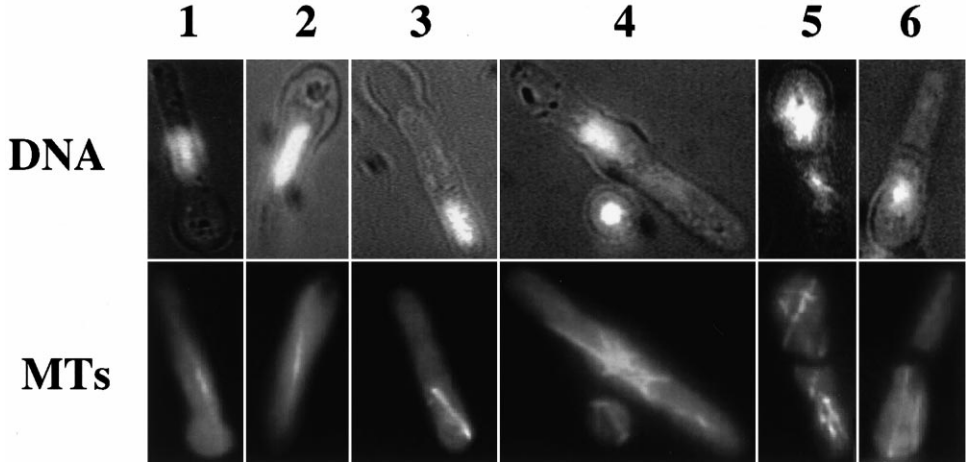

Fig. 3. Cells disrupted for *pbh1*$^+$ do not elongate their spindles to full length. *pbh1* null spores were germinated at 30°C in appropriate media for 20 h, fixed with methanol and stained with DAPI and anti-tubulin antibodies, TAT1, to visualize DNA and microtubules (MTs) respectively.

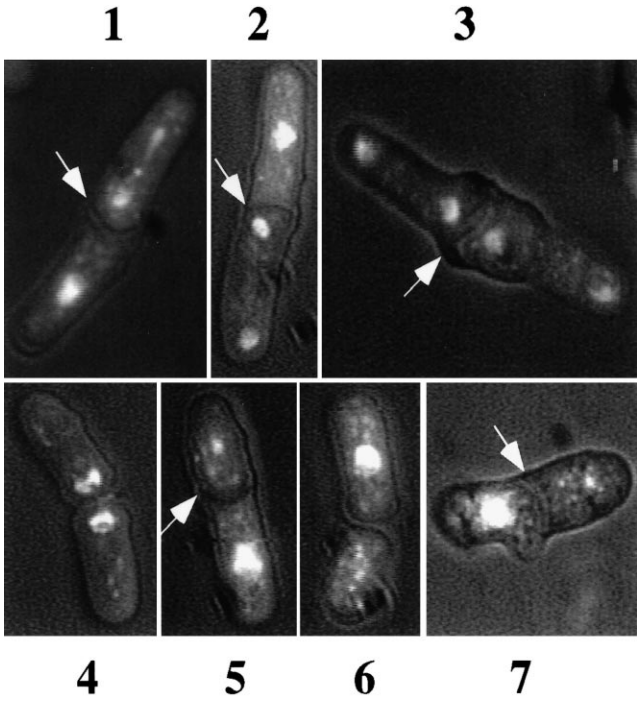

Fig. 4. Overproduction of Pbh1p causes mitotic and cytokinetic defects. Wild-type cells containing pREP3X-*pbh1*$^+$ were grown in EMM lacking thiamine at 30°C for 20 h, fixed with formaldehyde and stained with DAPI to visualize DNA. The arrows indicate the division septum.

4 shows a classical 'cut' phenotype, cells 5, 6, and 7 show unequal segregation of chromosomes. These data indicate that the levels of Pbh1p are important for proper regulation of chromosome segregation and cytokinesis.

4. Discussion

BIR domains were first identified in a class of baculoviral proteins (e.g. Op-IAP), which played a role in prevention of apoptosis of the insect cell line upon viral infection [2,3]. Recently, cDNA cloning and genome sequencing projects by several groups have identified several cellular BIR domain containing proteins. BIR domain proteins are thought to inhibit apoptosis, by preventing the action of caspases, cysteine-aspartyl proteases that are essential for apoptosis [14,15]. Interestingly, even though caspases have not been found in yeasts, proteins containing BIR domains have been identified in both *S. cerevisiae* and *S. pombe*. Thus, BIR domain containing proteins might play important roles in cell regulation in addition to controlling apoptosis. Our analysis of the *S. pombe* BIR domain containing protein, Pbh1p, has established a role for this protein in the regulation of mitosis. Cells deleted for Pbh1p display defects in mitosis and exhibit phenotypes reminiscent of those seen in the 'cut' mutants.

At least two major classes of 'cut' mutants have been described from previous studies [16]. One group, containing *cut4*, *cut9*, and *nuc2*, arrest with highly condensed chromosomes and a short mitotic spindle. A second group of cut mutants, comprising *cut3*, *cut14* and *top2*, arrest with uncondensed chromosomes, although spindle elongation is not affected. Based on the analysis of the *pbh1* null mutant phenotype, *pbh1*$^+$ belongs to the second category, in that chromosome condensation is affected. However, full-length mitotic spindles, as seen in wild-type cells and mutants such as *cut3* [17], are not found in *pbh1* null mutants. The reason for this difference is currently unclear. Isolation and characterization of temperature-sensitive *pbh1* mutants and analysis of synchronous cell cultures might resolve if spindles do elongate in *pbh1* mutants.

Analysis of the amino acid sequence of Pbh1p revealed the presence of seven potential phosphorylation sites for the Cdc2p kinase. Given that Pbh1p is required for mitosis and that the activity of Cdc2p kinase is essential for mitosis and peaks at mitosis [6], it is likely that Pbh1p is a substrate of Cdc2p kinase. Generation and analysis of *pbh1* mutants that mimic constitutively phosphorylated or dephosphorylated forms should help clarify the role of phosphorylation in the regulation of Pbh1p function.

Overexpression of Pbh1p leads to defective mitosis and cytokinesis. Thus, regulation of Pbh1p levels is important for mitosis and cytokinesis. Given that *pbh1* null mutants are capable of performing cytokinesis, it is currently unclear if the defect in cytokinesis is a direct effect of overproduction of Pbh1p or an indirect effect of altered mitotic exit. However, it is also possible that the cytokinesis role of Pbh1p is redundant with an as yet unidentified protein(s), whereas its mitotic role is indispensable.

Acknowledgements: We wish to thank Dr. Michael Hengartner for pointing out to us the existence of a BIR domain protein in *S. pombe*. Many thanks are due to all members of the IMA-yeast laboratories, and in particular Mr. Kelvin Wong, Dr. Jianhua Liu, and Dr. Naweed Naqvi, for their encouragement, advice, and critical reading of the manuscript. This work was funded by research funds from the National Science and Technology Board, Singapore.

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