The fission yeast γ -tubulin complex is required in G₁ phase and is a component of the spindle assembly checkpoint

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Microtubule polymerization is initiated from the microtubule organizing centre (MTOC), which contains the γ -tubulin complex. We have identified fission yeast Alp4 and Alp6, which are homologues of the γ -tubulin-interacting proteins Sc.Spc97/Hs.Gcp2 and Sc.Spc98/Hs.Gcp3, respectively. The size of the fission yeast γ -tubulin complex is large (>2000 kDa), comparable to that in metazoans. Both Alp4 and Alp6 localize to the spindle pole body (SPB) and also to the equatorial MTOC. Temperature-sensitive (*ts*) *alp4* and *alp6* mutants show two types of microtubular defects. First, monopolar mitotic spindles form. Secondly, abnormally long cytoplasmic microtubules appear that do not stop at the cell tips and are still associated with the SPB. Alp4 function is required in G₁ phase and *ts* mutants become lethal before S-phase. *alp4* and *alp6* mutants are hypersensitive to the microtubule-destabilizing drug thiabendazole (TBZ) and show a lethal 'cut' phenotype in its presence. Furthermore, *alp4mad2* double mutants show an exaggerated multiple septation phenotype in TBZ. These results indicate that Alp4 and Alp6 may play a crucial role in the spindle pole-mediated checkpoint pathway.

Keywords: fission yeast/microtubules/MTOC/spindle checkpoint/ γ -tubulin

Introduction

In many eukaryotic cells, microtubule nucleation occurs at a specific structure called the microtubule organizing centre (MTOC; Pickett-Heaps, 1969). The MTOC plays a vital role not only in nucleating microtubules, but also in determining their polarity. The slow growing minus end is embedded within the MTOC, while the fast growing plus end radiates out into either the cytoplasm or the nucleus (Heidemann and McIntosh, 1980). MTOC structure varies considerably between different species. In animal cells, the centrosome, in particular the pericentriolar material (PCM), functions as the MTOC (Gould and Borisy, 1978; Kellogg *et al.*, 1994). In yeast, the equivalent structure is the spindle pole body (SPB).

Central to MTOC function is the γ -tubulin complex. γ -tubulin, a member of the tubulin superfamily, was originally identified genetically as an intergenic suppressor of a β -tubulin mutation in *Aspergillus nidulans* (Oakley and Oakley, 1989). Subsequent work in various systems both *in vivo* and *in vitro* has established that γ -tubulin is a

universal component of the MTOC, and plays a central role in microtubule nucleation (Oakley *et al.*, 1990; Horio *et al.*, 1991; Stearns *et al.*, 1991; Zheng *et al.*, 1991; Joshi *et al.*, 1992, 1993; Oakley, 1992; Felix *et al.*, 1994; Stearns and Kirschner, 1994; Schnackenberg *et al.*, 1998). γ -tubulin does not appear to exist in the cell on its own; instead it is part of a large complex (Stearns and Kirschner, 1994). This γ -tubulin complex in animal cells comprises an open ring structure of 25 nm diameter, called the γ -tubulin ring complex (γ TuRC), which exists in both the cytoplasm and the PCM (Moritz *et al.*, 1995, 1998; Zheng *et al.*, 1995) and functions as a minus end capping factor for microtubule nucleation (Keating and Borisy, 2000; Leguy *et al.*, 2000; Moritz *et al.*, 2000; Wiese and Zheng, 2000).

Genetic studies in budding yeast have been instrumental in identifying other components of the γ TuRC. Spc97 and Spc98 were identified as γ -tubulin-interacting proteins and shown to constitute the major components of the *Saccharomyces cerevisiae* γ -tubulin complex (Geissler *et al.*, 1996; Knop and Schiebel, 1997; Knop *et al.*, 1997). Homologues of Spc97 and Spc98 have subsequently been found in humans and are called Gcp2 and Gcp3, respectively (Martin *et al.*, 1998; Murphy *et al.*, 1998; Tassin *et al.*, 1998). The budding yeast counterpart of γ -tubulin, Tub4, which also plays an important role in microtubule organization in this organism, is more divergent from γ -tubulins in other organisms (<40% amino acid identity with metazoan γ -tubulins as opposed to 70% identity between them), and metazoan γ -tubulin is not capable of substituting for Tub4 function (Burns, 1995; Sobel and Snyder, 1995; Marschall *et al.*, 1996). Furthermore, the Tub4-containing complex is much smaller than the mammalian γ TuRC (200–250 kDa versus >2000 kDa; Stearns and Kirschner, 1994; Zheng *et al.*, 1995; Knop *et al.*, 1997).

The fission yeast γ -tubulin homologue Gtb1/Tug1 shares >70% identity with the vertebrate protein (Horio *et al.*, 1991; Stearns *et al.*, 1991) and, more importantly, human γ -tubulin can rescue the lethal *gtb1* deletion (Horio and Oakley, 1994). Gtb1 localizes to the SPB throughout the cell cycle and to the equatorial MTOC in post-anaphase (Horio *et al.*, 1991; Masuda *et al.*, 1992). Like most other eukaryotes, fission yeast microtubule organization alters dynamically during cell cycle progression, where interphase cytoplasmic arrays give way for the mitotic bipolar spindle (Hagan and Hyams, 1988). Despite the attractions of this system, no conditional mutants that are defective in the components of the γ -tubulin complex have been available until recently (Paluh *et al.*, 2000).

The spindle assembly checkpoint is a surveillance mechanism that ensures that paired chromatids do not segregate until the chromosomes are aligned properly along the mitotic spindle, and it plays a pivotal role in the maintenance of genome integrity and fidelity of chromo-

Fig. 1. Defective phenotypes of *ts alp4* and *alp6* mutants and the cellular localization of Alp4 and Alp6 at the MTOC. (A) Wild-type (left, HM123, Table II), *alp4-1891* (middle, DH1891) or *alp6-719* (right, DH719) cells were incubated at 36°C for 6 h and processed for immunofluorescence microscopy. Merged images of anti-tubulin staining (TAT-1, red) and nuclear staining (DAPI, blue) are shown. Abnormally segregated mitotic chromosomes are marked with arrows. (B) Localization of Alp4. Fluorescence from GFP (two left panels; Alp4-GFP, LV11) or immunofluorescence microscopy using anti-HA antibody (two right panels; Alp4-3HA, LV15) are shown. (C) Localization of Alp4-GFP during the cell cycle. Triple staining using DAPI (left), anti-Sad1 (the second panels), GFP (the third panels) and merged pictures (right) during the cell cycle are shown. Representative images from interphase (row 1), metaphase (row 2), anaphase (row 3), post-anaphase (row 4) and septated cells (row 5) are presented. In the rightmost corner, combined images are depicted, in which blue corresponds to chromosomal DNA, orange shows the merged images between Sad1 and Alp4, and green presents Alp4 at the equatorial MTOC (marked with an arrow in row 4). (D) 'Ring' structures of the equatorial MTOC (shown by arrows, Alp4-3HA). Anti-HA signals from a post-anaphase cell (corresponding to row 4) have been rotating around a vertical axis after observation under a confocal microscope. The bar indicates 10 μ m.

some separation (Hoyt *et al.*, 1991; Li and Murray, 1991; Lengauer *et al.*, 1998). Recent analysis has shed more light on the complexities of this regulatory mechanism. In yeast, two distinct pathways are operational, one is Mad2 dependent whilst the other is Bub2 (Cdc16 in fission yeast) dependent (Burke 2000; Cerutti and Simanis, 2000). The Mad2 pathway is believed to be involved in the regulation of kinetochore function, such that its structural component plays a role in the checkpoint system (e.g. budding yeast Ndc10; Tavormina and Burke, 1998). In contrast, how microtubule/spindle integrity is monitored

by the Bub2 pathway remains elusive, although recent analysis indicates that it regulates mitotic exit via nuclear positioning in budding yeast (Bardin *et al.*, 2000; Bloecher *et al.*, 2000; Pereira *et al.*, 2000) and septation/cytokinesis in fission yeast (Cerutti and Simanis, 2000).

We previously isolated a number of mutants that display temperature-sensitive (*ts*) defects in the maintenance of growth polarity control (*alp* loci, *altered polarity*; Radcliffe *et al.*, 1998). In line with the fact that fission yeast microtubules play a crucial role in the determination of growth polarity, many of the *alp*⁺ genes encode

conserved proteins that are required for microtubule function, including tubulins and cofactor homologues (Hirata *et al.*, 1998; Radcliffe *et al.*, 1998, 1999). In this study, we describe the characterization of Alp4 and Alp6, which are the fission yeast homologues of Spc97/Gcp2 and Spc98/Gcp3, respectively. We show that Alp4 and Alp6 are required for the regulation of both interphase microtubules and mitotic bipolar spindles. Importantly, these components execute their essential role in G₁ phase. Furthermore, we show that Alp4 and Alp6 are essential components of the spindle assembly checkpoint.

Results

ts alp4 and alp6 mutants are defective in microtubule organization and show growth polarity defects

ts alp4 and *alp6* mutants were isolated from a visual screen for growth polarity mutants (Radcliffe *et al.*, 1998). Morphological characterization showed that these three strains showed similar, if not identical, phenotypes at the restrictive temperature, i.e. a bent shape associated with 'cut' phenotypes (9–10%). Furthermore, immunofluorescence microscopy using anti- α -tubulin antibody showed that mutant cells incubated for 6 h at 36°C displayed abnormal microtubules, and nuclear DNA often became displaced from the centre of the cell and segregated aberrantly into two or three masses (Figure 1A). These observations indicate that Alp4 and Alp6 are required for microtubule organization and proper chromosome separation.

alp4⁺ and alp6⁺ encode conserved components of the γ -tubulin complex

A fission yeast genomic library was used to isolate genes that complemented the *ts alp4* and *alp6* mutations. Two different plasmids (pLV4-1 and pLV6-1) were isolated that complemented *alp4-1891* and *alp6-719*, respectively. Genetic linkage analysis indicated that the gene in pLV4-1 is *alp4⁺*, whilst that in pLV6-1 is *alp6⁺* (see Materials and methods). Nucleotide sequencing showed that *alp4⁺* and *alp6⁺* encode fission yeast homologues of universal components of the γ -tubulin complex, Gcp2 (human)/Spc97 (budding yeast) and Gcp3/Spc98, respectively (Schiebel, 2000). The identity between Alp4 and Gcp2 or Spc97 was 22% (32% if conservative changes are considered) and 11% (22%), whilst that between Alp6 and Gcp3 or Spc98 was 25% (39%) and 18% (32%), respectively, indicating that the fission yeast proteins are evolutionarily closer to vertebrates than budding yeast (Table I). In a similar manner to the vertebrate proteins (Martin *et al.*, 1998; Murphy *et al.*, 1998; Tassin *et al.*, 1998), Alp4 and Alp6 show a distant evolutionary relatedness to each other (data not shown), and probably evolved from a common ancestor.

The cellular localization of Alp4 and Alp6 at the MTOC

In order to examine the cellular localization of Alp4 and Alp6, the green fluorescent protein (GFP) gene was fused to the C-terminus of the chromosomal *alp4⁺* and *alp6⁺* genes. GFP tagging did not interfere with protein function as strains containing Alp4-GFP or Alp6-GFP grew as well

Table I. Homology of γ -tubulin-interacting proteins between fission yeast and other eukaryotes

	Gcp2	Dgrip84	Spc97
Alp4	22 (32)	18 (32)	11 (22)
Gcp2		27 (42)	11 (24)
Dgrip84			10 (22)
	Gcp3	Dgrip91	Spc98
Alp6	25 (39)	19 (32)	18 (32)
Gcp3		29 (44)	15 (28)
Dgrip91			15 (28)

The percentage identity or similarity (in parentheses) in amino acid sequence between γ -tubulin-interacting proteins from fission yeast (Alp4 and Alp6), human (Gcp2 and Gcp3), fly (Dgrip84 and Dgrip91) and budding yeast (Spc97 and Spc98) is shown.

as wild-type strains, and no morphological or mitotic defects were apparent. Signals from Alp4-GFP and Alp6-GFP were essentially the same (although the signal from Alp4-GFP was stronger than that from Alp6-GFP). Signals were seen either as a single or double spot around the nuclear periphery and/or as dot-like structures in the cell centre in exponentially growing Alp4-GFP cells (the left panels in Figure 1B). As this localization appeared similar to that of the SPB, double staining with both Alp4-GFP and anti-Sad1 antibodies was performed (Sad1 is an SPB component; Hagan and Yanagida, 1995). As shown in Figure 1C, in both interphase (rows 1) and mitotic cells (rows 2 and 3), Alp4-GFP co-localized precisely with Sad1 either as a single (row 1) or double spot (rows 2 and 3). Of particular interest were post-anaphase cells (row 4), in which the localization of Alp4-GFP differed from that of Sad1. At this stage, in addition to the SPBs, which are located on the side of each nucleus, an equatorial dot(s) of Alp4-GFP but not anti-Sad1 was clearly visible (arrow in row 4). After the completion of septum formation, this central dot(s) disappeared (row 5). These dots appeared to be identical to those seen with anti-Gtb1, which have been termed the equatorial MTOC and which generate the post-anaphase array during cytokinesis (Hagan and Hyams, 1988; Horio *et al.*, 1991; Hagan, 1998).

In order to observe the three-dimensional structure of the equatorial MTOC, confocal images were rotated around a vertical axis. It was found that these central signals are in fact not 'dots', but instead formed a 'ring' structure during the early stages of cytokinesis (arrow, Figure 1D). It has been reported previously that cytoplasmic microtubules also exist as 'equatorial rings' in the centre of the cell at the end of anaphase and during telophase (Pichová *et al.*, 1995). These results show that Alp4 and Alp6 are not only homologous to components of the MTOC in their amino acid sequence, but are also an integral part of the two fission yeast MTOCs that are functional during the mitotic cell cycle.

Physical and genetic interactions between Alp4, Alp6 and γ -tubulin

Immunoprecipitation experiments were performed to explore the physical interactions between Alp4, Alp6 and Gtb1. Haemagglutinin (HA) epitope-tagged strains

Fig. 2. Physical and genetic interactions between Alp4/Alp6 and γ -tubulin. (A) Identification of the *alp4*⁺ and *alp6*⁺ gene products. Immunoblotting was performed with anti-HA antibody against cell extracts prepared from an *Alp4-3HA* (lane 1, LV15) or *Alp6-3HA* strain (lane 2, LV16). Gtb1 (γ -tubulin) was used as a loading control. (B) Physical interaction between Alp4/Alp6 and γ -tubulin. Cell extracts were prepared from an *Alp4-3HA* strain (lanes 1 and 4, LV15), an *Alp6-3HA* strain (lanes 2 and 5, LV16) or an untagged wild type (lanes 3 and 6), and immunoprecipitation performed with anti-HA antibody (lanes 4–6). Precipitated proteins were detected with anti-HA or anti-Gtb1 antibody. Total cell extracts (corresponding to 1/50 amount used for immunoprecipitation) were also run (lanes 1–3). (C) Gel filtration chromatography. Soluble cell extracts were analysed by immunoblotting with anti-HA or anti-Gtb1 antibody. Total extract (10 μ g) was run in the far-left panel. Protein extracts from two strains (*Alp4-3HA* and *Alp6-3HA*) were loaded on separate columns, and separation profiles were superimposed according to a control pattern using anti-Gtb1 antibody. The positions of size markers (2000, 669 and 232 kDa) are also shown. (D) Suppression analysis by multicopy plasmids. *ts alp4* or *alp6* mutants were transformed with an empty vector or multicopy plasmids containing *alp4*⁺ or *alp6*⁺ (pUR-*Alp4* or pUR-*Alp6*, respectively), and transformants were streaked on rich plates and incubated at 36°C for 3 days.

(*Alp4-3HA* and *Alp6-3HA*) were constructed in a manner similar to GFP tagging. Immunoblotting using anti-HA antibody identified *Alp4-3HA* and *Alp6-3HA* on an SDS–polyacrylamide gel (Figure 2A). These gene fusions were also used to confirm that *Alp4* and *Alp6* localize to the MTOC. Immunofluorescence microscopy using anti-HA antibody showed the same localization patterns as those of GFP-tagged proteins (see the right two panels in Figure 1B). Immunoprecipitation experiments showed that *Alp4-3HA* and *Alp6-3HA* co-precipitated with Gtb1 (Figure 2B, lanes 4 and 5). It appeared that the amount of Gtb1 that was co-precipitated was not as much as the total protein (compare lanes 1 and 2 with lanes 3 and 4). It is possible that *Alp4-3HA* and *Alp6-3HA*, which form a complex with Gtb1, are less efficient than free proteins for precipitation with anti-HA antibody, or that a subpopulation of Gtb1 may exist independently of *Alp4* and *Alp6*.

In order to address the size of the γ -tubulin complex in fission yeast, gel filtration analysis was performed. As shown in Figure 2C, *Alp4*, *Alp6* and Gtb1 co-fractionate predominantly in a large complex (>2000 kDa). It should be noted that this large size is comparable to that in higher eukaryotes such as *Drosophila* and mammalian cells (Martin *et al.*, 1998; Murphy *et al.*, 1998). We have observed that a small amount of Gtb1 is also found in the smaller fractions (fractions 13–22), some of which still overlap with *Alp4* and *Alp6* (13–16). It is possible that, as in other eukaryotes, multiple forms of the complex may

exist in fission yeast, which vary in size (Akashi *et al.*, 1997; Moritz *et al.*, 1998).

We sought genetic interactions to substantiate the biochemical data that *Alp4*, *Alp6* and Gtb1 physically interact. As shown in Figure 2D, multicopy plasmids containing the *alp4*⁺ gene suppressed the defects of the *ts alp6-719* mutant. Taken together, these results show that *Alp4* and *Alp6* localize to the MTOC and constitute part of the γ -tubulin complex, and that the *alp4*⁺ and *alp6*⁺ genes interact genetically with *gtb1*⁺ and also with each other.

***alp4* mutants exhibit defects in the formation of bipolar mitotic spindles and maintenance of the length of cytoplasmic microtubules**

In order to characterize the *ts alp4* phenotypes more carefully, a culture of *alp4* cells was synchronized with respect to cell cycle progression by centrifugal elutriation and shifted to the restrictive temperature. Small early G₂ *alp4-1891* cells grown at 26°C were collected by elutriation and the culture was divided into two halves. One half was incubated at 26°C to monitor the degree of synchrony of cell cycle progression, whilst the other half was shifted up to 36°C to examine the phenotypes arising from incubation under the restrictive conditions. Samples were collected at 20 min intervals in order to measure viability and to use for immunofluorescence analysis of microtubules and the SPB.

Fig. 3. Alp4 is required for both the formation of mitotic bipolar spindles and the integrity of interphase cytoplasmic microtubules. (A and B) Centrifugal elutriation. Small early G₂ cells of an *alp4-1891* strain (DH1891) were collected by centrifugal elutriation and the cultures were divided into two parts, one part incubated at 26°C (A) and the other at 36°C (B). Samples were collected at 20 min intervals, and cell number (open diamonds in dark blue), septation index (open squares in black) and viability (open diamonds in red, the percentage of viable colonies) were measured. Immunofluorescence microscopy with anti-tubulin, anti-Sad1 antibody and DAPI was performed to examine the percentage of cells containing anaphase B nuclei (open circles in green) and abnormal mitotic DNA (open triangles in blue). (C) Abnormally long interphase microtubules in the *alp4-1891* mutant. Confocal microscopy was performed using synchronous interphase samples (220 min at 36°C) with anti-tubulin (red) and anti-Sad1 (green) antibodies. Merged images are shown. (D) Aberrant mitotic spindles. Characteristic cells showing monopolar-like spindles (320 min) are presented. (E) Displaced long interphase microtubules. Interphase-like cells with longer cytoplasmic microtubules after an aberrant mitosis (420 min) are shown. (F) Teal1 localization in the *alp4-1891* mutant. *alp4* mutants were incubated at 26°C for 3 h in the presence of 10 mM HU and shifted up to 36°C after washout of HU. After 2.5 h incubation, cells were fixed and processed for immunofluorescence microscopy using anti-tubulin (left) and anti-Teal1 (middle) antibodies. Merged images are shown on the right. (G) Defects in microtubules by overexpression of *alp4+*. Cells containing integrated *nmt1-alp4+* were grown in the absence of thiamine, and processed for immunofluorescence microscopy using anti-tubulin antibody. Images are from confocal microscopy. Cells from a wild-type control (left) and *alp4+* overexpression after 16 h (right) are shown. The bar indicates 10 μm.

Synchrony was high as the septation index was <0.5% at 0 min, reached 62% at 120 min (the first mitosis), dropped to 1% at 200 min and peaked again up to 47% at 300 min (the second mitosis) at 26°C (Figure 3A). At the restrictive temperature, defective phenotypes, including abnormal mitotic DNA (Figure 1A), became evident only during the second mitosis; the first mitosis appeared to occur normally (Figure 3B; note the normal mitotic pattern until 200 min). Cell viability remained high during the first 100 min when the septation index (equivalent to the population of cells that have passed mitosis) peaked, and then started to drop as the second cycle proceeded. It is of note that in fission yeast, G₁ phase is very short and S phase occurs almost simultaneously with nuclear division and

septation (Moreno *et al.*, 1991). This result, therefore, suggested that *alp4* mutants are committed to death before the second M phase.

Immunofluorescence microscopy at each time point enabled us to follow temporally the defective phenotypes of the *alp4* mutant and delineate the kinetics of alterations in microtubules. The first phenotype to be seen was the appearance of abnormally long cytoplasmic microtubules. In wild-type cells, the interphase microtubule cytoskeleton consists of several filamentous forms that extend along the long axis of the cell with varied lengths but never curl around the cell tip (Hagan, 1998). In contrast, in the *alp4* mutant, the majority of interphase cells had longer microtubules, which curved around the cell end (220 min,

Figure 3C). Whilst most of the microtubules in wild-type cells appear to be independent of the SPB (Hagan, 1998), these longer microtubules generally associated with or passed through the SPB. Upon entry into the second mitosis, chromosome separation often occurred, albeit only partially, and the SPB separated into two bodies. However, bipolar spindles were rarely observed, and, instead, aberrant 'monopolar'-like spindles were formed (320 min, Figure 3D). In these cells, spindles generally emanated from only one of the two SPBs.

The cell cycle continued despite these mitotic defects and cells exited mitosis as monopolar spindles disappeared and the septum cleaved the undivided nuclei to produce a 'cut' phenotype. At this stage, the interphase microtubules reappeared (420 min, Figure 3E). Again, like the earlier interphase time point (Figure 3C), the length of cytoplasmic microtubules was abnormal and they grew right around the end of the cell. These cytoplasmic microtubules were often formed only in one half of the cell, which corresponded to the side with the displaced nucleus (see Figure 1A). Taken together, this analysis established the notion that Alp4 is required for the formation of cell cycle-dependent microtubule structures. In interphase, it is required to maintain the length of cytoplasmic microtubules and, in mitosis, it is essential for the formation of bipolar spindles.

Localization of the end marker *Tea1* is not defective in *alp4* mutants

Abnormally long interphase microtubules have been reported previously in *tea1* mutants, which are defective in the cell end marker for growth polarity control (Mata and Nurse, 1997). In the absence of *Tea1*, which usually localizes to the cell end, as well as along microtubules and at their tips, cytoplasmic microtubules do not terminate at the cell end; instead they curve around the cell cortex. In order to examine whether or not the long microtubules observed in *alp4* mutants are attributable to a disfunction of *Tea1*, double immunofluorescence microscopy was performed with anti-*Tea1* and anti-tubulin antibodies. A synchronous culture was prepared using a hydroxyurea (HU) block and release method. Upon washout of HU and release to the restrictive temperature, elongated and curved microtubules were observed (Figure 3F). Co-staining with anti-*Tea1* antibody revealed that *Tea1* localization is not disturbed. *Tea1* localized to both the cell ends and the tips of microtubules. Therefore, the longer cytoplasmic microtubules are not ascribable to *Tea1* mislocalization, rather they might be due to defects in SPB function.

Longer interphase microtubules were also seen in cells in which *alp4*⁺ or *alp6*⁺ were overexpressed ectopically. The thiamine-repressible strong *nmt1*⁺ promoter was integrated into the chromosomal *alp4*⁺ and *alp6*⁺ loci just prior to the initiation codon. It was found that ectopic overexpression of *alp4*⁺ was toxic and inhibits colony formation on plates in the absence of thiamine (data not shown). Very few mitotic spindles (<0.5%, in contrast to 2–4% in exponentially growing wild-type culture) were observed after 24 h of overinduction. Instead, as in *ts* mutants, elongated interphase microtubules were seen (16 h after induction, Figure 3G). These results indicate that the protein levels or activities of Alp4 and Alp6 have

to be regulated correctly during the cell cycle to ensure the formation of functional interphase microtubules and mitotic spindles.

***Alp4* function is executed prior to S phase regardless of the fact that the mutant shows mitotic abnormalities in the subsequent M phase**

We were interested in the cell cycle stage at which Alp4 executes its essential function. The results of the previous elutriation analysis were somewhat unexpected, as, although the *alp4* mutant showed a variety of mitotic defects, these phenotypes appeared only in the second mitosis. In order to address the question of when Alp4 executes its essential role, centrifugal elutriation experiments were repeated with the following modifications; after elutriation, the cultures were kept at 26°C for various times (0, 40, 80, 100, 120, 140 and 160 min; Figure 4A), then shifted up to 36°C and the appearance of abnormal mitosis in each sample was followed. The results are summarized schematically in Figure 4B. It transpired from these manipulations that there is a critical time when Alp4 function is executed. Until 100 min when the mutant was finishing the first mitosis (Figure 4A), the defects appeared in the second mitosis, as in the case when the culture was shifted up at 0 min (Figure 4B). On the other hand, if the culture was shifted up at 140 min or later after the first mitosis was complete, the defective phenotypes started to appear upon entry into the third mitosis. Thus, it appeared that the crucial stage at which Alp4 function is executed occurs between 120 and 140 min, which is likely to correspond to G₁ phase. These results suggest that an Alp4 function has already been accomplished before S, G₂ and M phases, irrespective of the fact that *alp4* mutants show defective phenotypes only in the subsequent mitosis.

To examine more precisely the cell cycle timing of the requirement for Alp4 function, *alp4* mutant cells were synchronized at 26°C at different stages by methods other than elutriation. These included HU block and release (early S), and nitrogen deprivation and addition (G₁) experiments. Following synchronization, cultures were shifted up to 36°C and the appearance of defective mitoses and cell viability were monitored. Following release from an HU block at the restrictive temperature, *alp4* cells divided once normally and then died in the second mitosis (Figure 4C), with kinetics very similar to those of the previous elutriation experiments when cells were shifted to 36°C at early G₂ (Figure 3B). On the other hand, following nitrogen deprivation and addition, cells died in the first mitosis accompanied by the appearance of abnormal chromosome separation (Figure 4D). In addition, the *alp4* mutant became lethal only when the cell cycle was allowed to progress, as cells maintained viability under continued incubation in nitrogen-deprived medium at 36°C for 7 h (Figure 4D). From this, we conclude that the execution point of Alp4 occurs during the G₁ phase of the cell cycle.

***alp4* mutants lose viability before entry into M phase**

We were interested in which stage *alp4* mutants become irreversibly committed to mitotic defects and viability loss under restrictive conditions. The issue addressed was whether or not continued incubation after G₁ phase until

mitotic entry at the restrictive temperature was necessary for abnormal mitotic phenotypes and loss of viability. To answer this question, synchronous culture analysis was repeated using nitrogen starvation at the permissive temperature (26°C) and released to 36°C upon addition of nitrogen. Aliquots of the culture were removed at each time point and divided into four parts: the first part was used to determine DNA content by flow cytometry (FACS); the second was used to observe mitotic phenotypes by 4',6-diamidino 2-phenylindole (DAPI) and calcofluor; the third was spread directly on rich plates at 26°C to measure viability; and the fourth was shifted down to 26°C in liquid culture in order to monitor mitotic progression after transfer from restrictive to permissive conditions.

As shown in Figure 5A and B, this analysis indicated that viability loss already started after 2.5 h at 36°C (Figure 5A, closed boxes), at which point S phase had finished (Figure 5B). Entry into the subsequent mitosis occurred only after 4 h (Figure 5A, closed circles). Thus, there was a 1.5 h period in which viability started to decrease before entry into M phase. This result indicated that *alp4* mutants are committed to lethality before entry into M phase at the restrictive temperature. Furthermore, observation of the consequent lethal phenotypes of these cells following incubation in permissive conditions showed that mutant cells displayed an abnormal mitosis at 26°C, as in the case of continued incubation at 36°C (Figure 5A and see below).

Further support for the requirement for Alp4 function during G₁ came from the experiment using HU. In this case, HU was added to *alp4* mutants at the same time as cultures were shifted from the permissive to the restrictive temperature. After 4 h at 36°C, HU was washed out and the cultures were returned to 26°C. If the essential Alp4 function is executed during G₁, abnormal mitosis should be observed under permissive conditions at 26°C, once *alp4* mutants pass through G₁ at 36°C in the presence of HU. As expected, after 2 h incubation at 26°C upon washout of HU, abnormal mitotic cells with monopolar spindles were observed (40% among mitotic cells, Figure 5C). These results established the notion that in

the *alp4* mutant, when the essential function in G₁ is not accomplished, cells proceed irreversibly through a lethal mitosis with monopolar spindles even if they are shifted down to permissive conditions prior to entry into mitosis.

The *alp4* mutation does not affect the formation of the γ -tubulin complex but is deficient in its cellular localization to the SPB

We sought to clarify what is defective in the *alp4* mutant. As a first step in addressing this question, the size of the γ -tubulin complex was examined in the *alp4-1891* mutant. Soluble extracts were prepared from wild type and the

Fig. 4. Essential Alp4 function is executed in G₁ phase. (A) Elutriation centrifugation and shift-up at different times. Centrifugal elutriation was performed to collect small G₂ cells from an exponentially growing *alp4* mutant at 26°C, and the synchronized cells continued to grow at 26°C. After various incubation times at 26°C (shown by arrows, 0, 40, 80, 100, 120, 140 and 160 min), the cultures were shifted up to 36°C. The septation index at 26°C is shown (up to the third mitosis). (B) Appearance of cells showing the defective mitosis at different time points. The percentage of cells that show aberrant mitosis after the first (black column), second (grey) or third mitosis (hatched) is plotted. (C) HU block and release experiments. The cultures of *alp4* mutant grown at 26°C in rich medium containing 10 mM HU for 3 h were shifted up to 36°C after washing out the HU. The septation index (open squares in black), viability (open diamonds in red), anaphase B nuclei (open circles in green) and abnormal mitotic DNA (open triangles in blue) at 36°C were examined as in Figure 3A and B. (D) Nitrogen deprivation and addition experiments. *alp4* (LV6) cells starved of nitrogen for 7 h at 26°C were divided into two parts: to one half, fresh rich medium was added and shifted up at 36°C, whilst the other half was kept in nitrogen-deprived medium and shifted up at 36°C. Symbols are the same as in (C) except that viability under nitrogen deprivation at 36°C (crosses in light blue) is also shown.

Fig. 5. *alp4* mutants become irreversibly lethal before entry into mitosis. (A) The timing of lethality. Nitrogen-starved, G_1 -synchronized *alp4* mutants at 26°C were shifted to 36°C upon addition of nitrogen. Samples were taken every 30 min, and viability loss (shaded boxes) and percentage of septated cells (closed circles) were measured. At each time point, aliquots were shifted down to 26°C and the percentage of cells that progressed through an abnormal mitosis (hatched) was counted. (B) DNA content upon nitrogen addition. Samples used in (A) were fixed and processed for FACS analysis. (C) Appearance of monopolar spindles under permissive conditions. *alp4* mutant cells grown at 26°C were shifted to 36°C and HU (10 mM) was added simultaneously. After 4 h incubation, HU was washed out, and the cultures were returned to 26°C. Samples were then taken at different time points for immunofluorescence microscopy with anti-tubulin antibody (red) and Cut12-GFP (green, an integral SPB protein; Bridge *et al.*, 1998). The figure (confocal microscopy) shows representative abnormal mitotic cells that have monopolar spindles (2 h at 26°C). The bar indicates 5 μ m.

alp4-1891 mutant, which had been incubated at the restrictive temperature for 6 h, and gel filtration was performed. As shown in Figure 6A, the majority of γ -tubulin still exists in a large complex, although, compared with wild type, there appears to be more γ -tubulin in the smaller fractions. This result suggests that the γ -tubulin complex is not significantly compromised in the *alp4* mutant.

To address further the possible defects in the *alp4* mutant, the cellular localization of γ -tubulin was examined. A synchronous culture was prepared using an HU block and the cultures were released to 36°C upon washout of HU. Affinity-purified anti-Gtb1 antibody stained both SPBs and the equatorial MTOC at 26°C (Figure 6B, left), as reported previously (Horio *et al.*, 1991). In contrast, in the *alp4* mutants at 36°C, γ -tubulin localized normally in the first mitosis when viability is high (90 min); however,

it failed to localize almost completely in the second cycle (300 min). Therefore, the *alp4-1891* mutant appears to be able to assemble the γ -tubulin complex but is defective in its localization to the SPB.

Alp4 and Alp6 are required for spindle checkpoint control

As shown earlier (see Figure 3B–E), *alp4* and *alp6* mutants incubated at the restrictive temperature, despite undergoing an abnormal mitosis with microtubular defects, did not display a typical ‘cdc’ arrest; instead they passed through the later mitotic stages such as septation and cytokinesis. Is there any functional link between Alp4/Alp6 and the spindle assembly checkpoint pathways? In order to answer this question, we examined the following points in the *alp4* mutants: (i) sensitivity to microtubule-destabilizing drugs; (ii) the ability of the *alp4* mutant to activate the spindle assembly checkpoint; and (iii) the involvement of Alp4 in the spindle checkpoint pathway itself. As shown in Figure 7A, it was found that both *alp4* and *alp6* mutants were hypersensitive to TBZ even at the permissive temperature. The hypersensitivity to TBZ appeared not to be attributable to simple structural deficiencies of microtubules, as the addition of TBZ to *alp4* mutants resulted in a sharp drop in viability, with cells displaying a ‘cut’ phenotype at 26°C instead of a mitotic arrest (Figure 7B). In addition, following an HU block at 26°C and release to 36°C, the *alp4* mutant also lost viability immediately on entry into the first mitosis in the presence of TBZ (Figure 7C). This is in sharp contrast to the situation when this strain was released to 36°C in the absence of this drug (viability loss in the second cycle). Thus, *alp4* (and *alp6*) is (are) deficient in some aspect of the surveillance mechanisms of spindle/microtubule integrity.

Alp4 functions in spindle checkpoint control independently of the Mad2 pathway

Next, a functional relationship between the Alp4- and Mad2-dependent checkpoints was explored. Sensitivity to TBZ was indistinguishable between *alp4* and *alp4mad2* mutants or between *alp6* and *alp6mad2* mutants at the permissive temperature (Figure 7A). None the less, the double mutant with *alp4-1891* showed novel phenotypes in the presence of TBZ at 26°C, in which multiply septated cells became greatly increased compared with the single mutants (Figure 8A and B). This indicated that Alp4 functions in the spindle checkpoint independently of Mad2. However, after an HU block (26°C) and release (36°C) in the absence of the drug, the double mutant showed a reduced appearance of the abnormal phenotypes (Figure 8C), suggesting that the Mad2-dependent pathway somehow plays a role in the appearance of the defective phenotypes of the *alp4* mutants at 36°C. It should be noted that the *alp4mad2* mutant showed neither rapid viability loss nor a ‘cut’ phenotype at 36°C in the first mitosis in the absence of TBZ, which is in clear contrast to the lethal phenotypes in the presence of this drug (compare Figures 8C with 7B and C). This indicated that there is no Mad2-dependent mitotic delay in the *alp4* mutant. Taking these results together, we conclude that Alp4 plays a role in the spindle checkpoint pathway independently of, but related to, the Mad2-mediated pathway.

Fig. 6. *alp4* mutants are defective in γ -tubulin localization to the SPB without affecting the capability of complex formation with γ -tubulin. **(A)** Gel filtration chromatography in *alp4* mutants. Soluble cell extracts were prepared from wild type and *alp4-1891* mutants that were incubated at 36°C for 6 h, separated through a Superose-6 column and immunoblotted as in Figure 2C. **(B)** Cellular localization of SPB (Cut12-GFP) and γ -tubulin. *alp4* mutants in which *cut12*⁺ was tagged with GFP (LV25, Table II) were grown at 26°C in the presence of HU and shifted up to 36°C upon washout of HU. Immunofluorescence microscopy using affinity-purified anti-Gtb1 antibody was performed. At the same time, the location of Cut12 was determined by GFP. The bar indicates 10 μ m.

Discussion

In this study, we describe the identification and characterization of two fission yeast homologues of components of the universally conserved γ -tubulin complex. The results shed light on a crucial role for the γ -tubulin complex in microtubule integrity, in particular, formation of both mitotic bipolar spindles and interphase microtubules, and also in spindle assembly checkpoint control.

A large γ -tubulin complex in fission yeast: the size matters

Gel filtration chromatography showed that the size of the fission yeast complex is large and is almost indistinguishable from that in metazoans, and it is likely that the complex exists in multiple forms with varied sizes (Akashi *et al.*, 1997; Moritz *et al.*, 1998; Oegema *et al.*, 1999). Biochemical work in higher systems has detected several additional proteins comprising the γ TuRC besides γ -tubulin, Gcp2 and Gcp3 (Zheng *et al.*, 1995; Martin *et al.*, 1998; Murphy *et al.*, 1998; Fava *et al.*, 1999). Given the similar size of the γ -tubulin complex between fission yeast and animals, fission yeast should be a suitable organism in which to identify such novel factors.

The essential role for Alp4 and Alp6 is accomplished during G₁: the timing matters

Careful analysis performed with various synchronous cultures using *ts alp4* mutants led us to conclude that Alp4 has already executed its essential role by S phase, and that the loss of viability occurred long before entry into the subsequent mitosis. What is the essential Alp4-dependent event that occurs during G₁? One possibility is that the recruitment of the γ -tubulin complex on to the SPB,

possibly coupled with the duplication of the SPB, takes place during G₁ in wild-type cells. In the *ts alp4* mutants, this process may be defective, rather than the inability to play a role in microtubule nucleation *per se*. The Alp4 mutant protein could remain functional if it is recruited on to the SPB sometime in G₁ at the permissive temperature. The fact that assembly of the γ -tubulin complex itself is not impaired in *ts alp4* mutants suggests that it may be its recruitment to the SPB that is defective.

Reports on the timing of duplication (not separation) of the fission yeast SPB have been inconclusive (McCully and Robinow, 1971; King and Hyams, 1982; Kanbe *et al.*, 1990; Ding *et al.*, 1997). Recent careful analysis, however, suggests that SPB duplication occurs during G₁ (S.Uzawa and W.Z.Cande, personal communication). The fact that the *alp4* mutant is still capable of nucleating a mitotic spindle from one SPB could be explained by γ -tubulin complexes having been retained from the previous cycle, albeit that they were not detectable by immunofluorescence microscopy. There is evidence for non-equivalence of the two SPBs, e.g. the appearance of monopolar mitotic spindles in *ts cut12* mutants that are defective in an SPB component (Bridge *et al.*, 1998) and the asymmetrical localization of the GTPase-activating proteins (Cdc16 and Byr4) and the effector (Cdc7) for the Spg1-GTP-binding protein to only one of the duplicated SPBs (Cerutti and Simanis, 2000). It should be noted that these proteins are all involved in the SPB-mediated spindle checkpoint pathway (see below).

The role of the γ -tubulin complex in interphase microtubule formation: the length matters

In wild type, cytoplasmic microtubules exist as several distinct filaments, most of which do not associate with the

Fig. 7. Alp4 and Alp6 are required for the spindle checkpoint pathways. (A) Hypersensitivity of *alp4* and *alp6* mutants to thiabendazole. The *alp4-1891* mutant containing plasmids carrying *alp4⁺* (wild-type equivalent), *mad2* deletion, *alp4-225*, *alp4-1891*, *alp4-1891mad2*, the *alp6-719* mutant containing plasmids carrying *alp6⁺* (wild-type equivalent), *alp6-719* and *alp6-719mad2* strains were spotted on to rich plates (-TBZ, right) or plates containing 20 μ g/ml thiabendazole (+TBZ, left) as serial dilutions (10^6 cells in the top row and then diluted 10-fold in each subsequent spot below) and incubated at 30°C. (B) Loss of viability in the presence of TBZ at the permissive temperature. *alp4* mutants were grown in rich medium at 26°C and TBZ (50 μ g/ml) was added. The percentage of 'cut' cells (closed squares) and the viability (open squares) were measured. (C) Loss of viability in the presence of TBZ at the restrictive temperature. *alp4* mutants were grown in rich medium in the presence of HU at 26°C for 3 h and shifted to 36°C upon washout of HU and addition of TBZ (50 μ g/ml). The percentage viability with (open squares) or without (open circles) addition of TBZ is also shown.

SPB (Hagan and Hyams, 1988). In contrast, *alp4* mutant cells exhibit the following two abnormalities. First, one or two greatly elongated microtubule bundles are seen. Secondly, these long microtubules generally associate

Fig. 8. Alp4 functions independently of but is related to the Mad2 pathway in spindle checkpoint control. (A) Multiple septation phenotypes of the *alp4mad2* double mutant in the presence of TBZ. *alp4-1891* (black), *alp4mad2* (hatched) and *mad2* (dotted) mutants were grown in rich medium at 26°C and synchronized with HU (10 mM). After 3 h incubation, HU was washed out and TBZ was added, and the culture was monitored for 4 h. The percentage of septating cells displaying multiple septa is shown. (B) Cell morphology in the presence of TBZ. Typical cell morphology stained with calcofluor is shown (top, *alp4-1891*; middle, *alp4mad2*; bottom, *mad2*). The bar indicates 10 μ m. (C) Progression through mitosis of the double mutant at the restrictive temperature. *alp4* single (squares) or *alp4mad2* double (circles) mutants were blocked with HU for 3 h at 26°C and released into HU-free medium at 36°C. The percentages of abnormal mitotic cells (closed symbols) and anaphase B nuclei (open symbols) were counted.

with or pass through the SPB. We show that the localization of the end marker Tea1 is not abolished in the *alp4* mutant. This suggests that the longer cytoplasmic microtubules may arise due to defects in microtubule organization at the SPB rather than a failure in Tea1-dependent growth polarity control.

It is possible that in the *alp4* mutant, by interacting with the SPB, these interphase microtubules become more stabilized than wild-type forms. Thus, Alp4 and Alp6 may play an important role in both the maintenance of the length and the orientation of cytoplasmic microtubules. A

Table II. Strain list

Strains	Genotypes	Derivations
HM123	<i>h⁻leu1</i>	our stock
DH225	<i>h⁻leu1alp4-225</i>	our stock
DH719	<i>h⁻leu1alp6-719</i>	our stock
DH1891	<i>h⁻leu1alp4-1891</i>	our stock
AE148	<i>h⁻leu1ura4mad2::ura4⁺</i>	from Dr Tomohiro Matsumoto
1356	<i>h⁺leu1ura4cut12⁺-GFP-ura4⁺</i>	from Dr Iain M.Hagan
LV1	<i>h⁻leu1ura4alp4-1891</i>	this study
LV2	<i>h⁻leuura41alp4-225</i>	this study
LV3	<i>h⁻leu1ura4alp6-719</i>	this study
LV6	<i>h⁻alp4-1891</i>	this study
LV7	<i>h⁻alp6-719</i>	this study
LV11	<i>h⁻leu1alp4⁺-GFP-kan^r</i>	this study
LV12	<i>h⁻leu1alp6⁺-GFP-kan^r</i>	this study
LV15	<i>h⁻leu1alp4⁺-3HA-kan^r</i>	this study
LV16	<i>h⁻leu1alp6⁺-3HA-kan^r</i>	this study
LV21	<i>h⁻leu1nmt1-alp4⁺-kan^r</i>	this study
LV22	<i>h⁻leu1nmt1-alp6⁺-kan^r</i>	this study
LV23	<i>h⁻leu1ura4alp4-1891mad2::ura4⁺</i>	this study
LV24	<i>h⁻leu1ura4alp6-719mad2::ura4⁺</i>	this study
LV25	<i>h⁻leu1ura4alp4-1891cut12⁺-GFP-ura4⁺</i>	this study

similar phenotype displaying longer microtubules has been observed in mutations in the γ -tubulin gene in budding yeast (Marschall *et al.*, 1996; Spang *et al.*, 1996) and recently in fission yeast (Paluh *et al.*, 2000). Defective phenotypes arising from ectopic overproduction of Alp4 appear similar to the mutant in terms of interphase microtubule morphology. This supports a role for these proteins in the maintenance of interphase microtubule integrity.

It remains to be determined how interphase microtubules are generated and organized. It is possible that they originate at the MTOC (SPB and/or the equatorial MTOC) and are then released into the cytoplasm. Alternatively, the nucleation of cytoplasmic microtubules could be independent of the MTOC, but still require some interaction with it. Alp4 and Alp6 may be necessary for the liberation of cytoplasmic microtubules from the MTOC by some biochemical processes such as microtubule-severing reactions. Microtubule-nucleating activity is under cell cycle control in fission yeast (Masuda and Shibata, 1996), and it is now important to address the molecular mechanisms underlying cell cycle-dependent regulation of MTOC function.

The role of the γ -tubulin complex in spindle checkpoint control: the place matters

The other significant finding in this study is the involvement of Alp4 and Alp6 in the spindle assembly checkpoint pathways. *alp4* and *alp6* mutants do not activate the Mad2-dependent checkpoint as other mitotic mutants do (e.g. mutations in β -tubulin encoding *nda3* or kinesin-encoding *cut7*; He *et al.*, 1997; Kim *et al.*, 1998). Instead, Alp4 and Alp6 are required for the activation of the spindle checkpoint pathway *per se*. This is a unique character because, in addition to checkpoint control, Alp4 and Alp6 play an essential role as structural components involved in microtubule/spindle biogenesis. The reason why *alp4* mutants do not activate the Mad2 pathway might be attributable to the location of Alp4 and Alp6. Analysis from several organisms suggests that the Mad2 pathway

monitors the state of kinetochores or kinetochore–spindle interactions (Taylor and McKeon, 1997; Burke, 2000). As Alp4 and Alp6 localize to the SPB rather than the kinetochores, kinetochore–spindle interactions might not be compromised, resulting in a failure to activate the Mad2-dependent checkpoint.

It has become clear that spindle assembly checkpoint control bifurcates, and both kinetochore- and the SPB-mediated pathways exist (Burke, 2000). The kinetochore checkpoint is believed to arrest the cells in metaphase by preventing sister chromatid segregation, whilst the SPB-mediated pathway is involved in the inhibition of mitotic exit, which in fission yeast involves septation and cytokinesis (Hwang *et al.*, 1998; Kim *et al.*, 1998; Cerutti and Simanis, 2000). The fact that the *alp4mad2* double mutant causes an exaggeration of the TBZ-sensitive phenotype at 26°C suggests that these two proteins may function independently in their response to this drug. Given the localization of Alp4 and Alp6 to the SPB, it is possible that Alp4 is involved in the SPB-mediated pathway. Unlike the kinetochore-mediated pathway, in which most of the components are conserved from yeasts to humans, components involved in the SPB checkpoint pathway so far have only been identified in yeast (e.g. the aforementioned Cdc16/Bub2, Byr4/Bfa1, Spg1/Tem1 and Cdc7/Cdc15). Given the universal conservation of the γ -tubulin complex, we propose that a centrosome- and γ -tubulin complex-dependent checkpoint is operational in vertebrates, in which Gcp2 and Gcp3 may play a crucial role.

Materials and methods

Strains, media and genetic methods

Strains used in this study are listed in Table II. YPD (2% dextrose, 2% polypeptone and 1% yeast extract) and YE5S were used as rich media, and modified synthetic EMM2 was used as minimal medium. The standard methods were followed as described (Moreno *et al.*, 1991).

Cloning of the *alp4⁺* and *alp6⁺* genes

A *Schizosaccharomyces pombe* genomic library in pUR19 (Barbet *et al.*, 1992) was used for the isolation of genes that complemented the ts *alp4-1891* and *alp6-719* mutants (LV1 and LV3, respectively, Table II). Thirty (for *alp4-1891*) and seven (for *alp6-719*) plasmids were isolated independently. Restriction enzyme mapping showed that they were classified into two (pLV4-1 and -2) and three different plasmids (pLV6-1, -2 and -3), respectively. pLV4-1 and pLV4-2 contained overlapping insert DNA, as did pLV6-1, pLV6-2 and pLV6-3. The identity of the two cloned genes as *alp4⁺* and *alp6⁺*, respectively, was confirmed by genetic crosses between tagged strains and original mutants as follows. Random spore analysis between an Alp4-3HA strain (LV15, kanamycin resistance as a marker, Table II) and a ts *alp4-1891* strain, or between an Alp6-3HA strain (LV16, Table II) and a ts *alp6-719* strain, showed that the gene that was isolated in pLV4-1 and pLV6-1 was most likely to be derived from the *alp4⁺* and *alp6⁺* loci, respectively (no recombinants, namely Kan^rTs⁻ or Kan^sTs⁺, were obtained from >10³ haploid segregants).

Nucleic acid preparation and manipulation

Enzymes were used as recommended by the suppliers (New England Biolabs). Nucleotide sequence data reported herein are in the DDBJ/EMBL/Genbank databases under accession Nos AB026664 (*alp4⁺*) and AB040811 (*alp6⁺*).

Gene disruption

The *alp6⁺* gene was deleted using PCR-generated fragments (Bähler *et al.*, 1998). Dissection of asci from heterozygous diploid cells showed that the *alp6⁺* gene is essential for cell viability, as two viable and two non-viable spores were obtained from 20 tetrads dissected and viable colonies were Ura⁻. Microscopic observation of haploid cells germinated from spores deleted for *alp6⁺* showed morphological defects similar to those of ts mutants.

Overexpression and C-terminal epitope tagging by chromosomal integration

The thiamine-repressible strong *nmt1* promoter was integrated in the genome in front of the initiator ATG of the *alp4⁺* and *alp6⁺* genes by a PCR-based gene targeting method (LV21 and LV22, Table II; Bähler *et al.*, 1998). C-terminal tagging with GFP or HA epitope was also performed using a PCR-generated fragment.

Synchronous culture

Centrifugal elutriation was performed using an elutriator rotor (JE-5.0, Beckman Instruments) as described previously (Moreno *et al.*, 1991). For HU block-release experiments, HU (10 mM) was added to exponentially growing cells at 26°C, incubated for 3 h, filtered, resuspended in HU-free rich medium and cultured at 36°C. Nitrogen deprivation and release experiments were performed using a prototrophic strain (LV6). EMM2 liquid medium lacking nitrogen (EMM2-N) was used and cells were starved in this medium for 7 h at 26°C. The medium was then washed out and replaced with fresh complete medium and the cultures were shifted up to 36°C. Samples were collected at each time point for immunofluorescence microscopy, the measurement of septation index and nuclear staining.

Gel filtration chromatography

Soluble protein extracts were prepared in buffer A (20 mM Tris-HCl pH 7.5, 20% glycerol, 0.1 mM EDTA, 1 mM mercaptoethanol, 5 mM ATP plus a cocktail of inhibitors; Sigma). Gel filtration chromatography was performed on a Superose-6 column by FPLC (Pharmacia Biotech). The column was equilibrated with two column volumes of buffer A containing 100 mM NaCl. To determine molecular weight, a parallel column was run with standards consisting of dextran (2000 kDa), thyroglobulin (669 kDa) and α -amylase (232 kDa). Fractions (50 μ l each) were separated by SDS-PAGE on 10% gels, and fractionated proteins were detected with individual antibodies.

Immunochemical assays

Affinity-purified rabbit polyclonal anti-Gtb1 and anti-Tea1 antibodies were a gift from Drs Hirohisa Masuda (Masuda and Shibata, 1996) and Manuel Arellano (Mata and Nurse, 1997), respectively. Rabbit polyclonal anti-Sad1 antibody was obtained from Dr Iain Hagan (Hagan and Yanagida, 1995). Mouse monoclonal anti- α -tubulin antibody (TAT-1) was provided by Dr Keith Gull. Mouse monoclonal anti-HA antibody (16B12) and anti- γ -tubulin antibody were purchased from BAbCO Ltd and Sigma, respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (Bio-Rad Laboratories) and a

chemiluminescence system (ECL, Amersham) were used to detect bound antibody. Fission yeast whole-cell extracts were prepared using glass beads to disrupt cells as described before (Radcliffe *et al.*, 1999). For immunoprecipitation, 2 mg of total protein extracts were used.

Indirect immunofluorescence microscopy

Cells were fixed with methanol, and primary antibodies (TAT-1, 1/50; anti-HA, 1/1000; anti-Sad1 antibody, 1/15; or anti-Gtb1 antibody, 1/50) were applied, followed by Cy3-conjugated goat anti-rabbit IgG (Sigma) or fluorescein-linked sheep anti-mouse IgG (Amersham). Immunofluorescence images were viewed with a chilled video-rated CCD camera (model C5985, Hamamatsu) connected to a computer (Apple Power Macintosh G3/400) and processed by use of Adobe® Photoshop (version 4). A confocal microscope LSM510 (Zeiss Co.) was also used.

Acknowledgements

We thank Drs Manuel Arellano, Keith Gull, Iain M.Hagan, Hirohisa Masuda and Tomohiro Matsumoto for providing antibodies and strains (anti-Tea1, TAT-1, anti-Sad1 and anti-Gtb1 antibody, and a *mad2* deletion and a Cut12-GFP-tagged strain) used in this study. We are indebted to Dr Iain M.Hagan who generously instructed us how to perform elutriation centrifugation, to Dr Gohta Goshima for stimulating discussion, and to W.Zac Cande and Satoru Uzawa for communicating results prior to publication. We thank Drs W.Zac Cande, Iain M.Hagan, Paul Nurse and Janet L.Paluh for critical reading of the manuscript and useful suggestions. This work is supported by the ICRF and an HFSP research grant.

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*Received April 7, 2000; revised September 4, 2000;
accepted September 22, 2000*