Functional Dissection of the γ -Tubulin Complex by Suppressor Analysis of *gtb1* and *alp4* Mutations in *Schizosaccharomyces pombe*

Yoshie Tange,* Akiko Fujita,[†] Takashi Toda[†] and Osami Niwa*^{*,1}

*Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan and [†]Cancer Research UK, London WC2A 3PX, United Kingdom

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ABSTRACT

In fission yeast, γ -tubulin (encoded by the *gtb1*⁺ gene), Alp4 (Spc97/GCP2), and Alp6 (Spc98/GCP3) are essential components of the γ -tubulin complex. We isolated *gtb1* mutants as allele-specific suppressors of temperature-sensitive *alp4* mutations. Mutation sites in *gtb1* mutants and in several *alp4* alleles were determined. The majority of substituted amino acids were mapped to a small area on the predicted surface of the γ -tubulin molecule that might directly interact with the Alp4 protein. The cold sensitivity of γ -tubulin mutants was almost completely suppressed by an α -tubulin mutation and partially suppressed by a low concentration of thiabendazole, a microtubule assembly inhibitor. Other *gtb1* mutants had increased resistance to this drug. Gel-filtration and immunoprecipitation analyses suggested that the mutant γ -tubulin formed an altered γ -tubulin complex with increased stability compared to wild-type γ -tubulin. In most *gtb1* mutants, sexual development was impaired, and aberrant asci that contained an irregular spore shape and number were produced. In contrast, spore formation was not appreciably damaged in some *alp4* and *alp6* mutants, even at temperatures where vegetative proliferation was substantially defective. These results suggested that the function of the γ -tubulin complex or the requirement of each component of the complex is differentially regulated between the vegetative and sexual phases of the life cycle in fission yeast. In addition, genetic data indicated intimate functional connections of γ -tubulin with several kinesin-like proteins.

UBIQUITOUS in eukaryotes, γ -tubulin is a key component of the microtubule-organizing center (MTOC), such as the centrosome in animal cells. γ -Tubulin has a crucial role in microtubule nucleation and in the anchoring of the minus end of microtubules to the MTOC and thereby contributes to the proper formation of mitotic spindles and cytoplasmic microtubular arrays (OAKLEY 2000; SCHIEBEL 2000; JOB *et al.* 2003). In the budding yeast *Saccharomyces cerevisiae*, γ -tubulin interacts with Spc97 and Spc98 to form a functional complex, which localizes at the spindle pole body (SPB, centrosome-equivalent organelle in yeast; KNOP *et al.* 1997; KNOP and SCHIEBEL 1997, 1998). Proteins with sequence similarity to Spc97 and Spc98 have been identified from a number of species, including human, frog, fly, nematode, plant, and fungi (MURPHY *et al.* 1998; OEGEMA *et al.* 1999; GUNAWARDANE *et al.* 2000; VARDY and TODA 2000; ERHARDT *et al.* 2002; HANNAK *et al.* 2002). These proteins contain conserved sequence motifs and are called gamma-ring proteins (GRIPs)/gamma-tubulin complex proteins (GCPs; MURPHY *et al.* 1998, 2001; GUNAWARDANE *et al.* 2000). A large form of γ -tubulin ring complex (γ -TuRC) has been identified in several organisms, including fly, frog, and human

(MORITZ *et al.* 1995, 2000; ZHENG *et al.* 1995; MURPHY *et al.* 1998). Upon incubation under high-salt conditions, a smaller complex is released from the *Drosophila* γ -TuRC. The γ -tubulin small complex (γ -TuSC) consists of Dgrip83, Dgrip91, and γ -tubulin and is thought to be structurally equivalent to the yeast Spc97/Spc98/ γ -tubulin complex (OEGEMA *et al.* 1999). *In vitro*, γ -TuSC is capable of nucleating microtubules, but much less efficiently than γ -TuRC (OEGEMA *et al.* 1999). Genetic studies in several lower eukaryotes revealed that the nucleation of microtubules is not the sole function of γ -tubulin *in vivo* but that it is likely involved in other aspects of microtubule dynamics, including the regulation of spindle elongation, mitotic checkpoints, and cytoplasmic microtubule stability (PALUH *et al.* 2000; VOGEL and SNYDER 2000; HENDRICKSON *et al.* 2001; JUNG *et al.* 2001). How γ -tubulin protein achieves these functions and how the γ -tubulin complex is regulated remain to be determined.

γ -Tubulin in the fission yeast *Schizosaccharomyces pombe* is encoded by *gtb1*⁺ (also known as *tug1*⁺) and is essential for viability (HORIO *et al.* 1991; STEARNS *et al.* 1991). Fission yeast carries apparent homologs of Spc97 and Spc98 in *S. cerevisiae*, respectively, encoded by *alp4*⁺ and *alp6*⁺ (VARDY and TODA 2000). As anticipated from the sequence similarity, biochemical as well as genetic analyses demonstrated that Alp4/Alp6 interacts with γ -tubulin and localizes at the MTOC in fission yeast

¹Corresponding author: Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan.
E-mail: niwa@kazusa.or.jp

TABLE 1
Strains used in this study

Strain	Genotype	Reference/Source
HM123	<i>h⁻ leu1</i>	Laboratory stock
HM487	<i>h⁺</i>	Laboratory stock
HM753	<i>h⁺ his2 leu1 ura4 alp4-1891</i>	VARDY and TODA (2000)
HM754	<i>h⁺ his2 leu1 ura4 alp6-719</i>	VARDY and TODA (2000)
HM760	<i>h⁻ leu1 ura4 alp4-B7</i>	This study
HM765	<i>h⁻ leu1 ura4 alp4-S5</i>	This study
HM766	<i>h⁻ leu1 ura4 alp4-S7</i>	This study
YT106	<i>h⁹⁰ mei1-102 leu1</i>	Laboratory stock
W549	<i>h⁻ alp6-719</i>	This study
W601	<i>h⁺ his2 leu1 alp4-1891 gtb1-93</i>	This study
W604	<i>h⁻ leu1 gtb1-93</i>	This study
W607	<i>h⁹⁰ mei1-102 leu1 gtb1-93</i>	This study
W609	<i>h⁺ gtb1-93</i>	This study
W665	<i>h⁹⁰ mei1-102 leu1 gtb1-22</i>	This study
W671	<i>h⁹⁰ mei1-102 leu1 gtb1-29</i>	This study
W705	<i>h⁻ leu1 alp4-1891</i>	This study
W711	<i>h⁺ gtb1-22</i>	This study
W714	<i>h⁺ gtb1-85</i>	This study
W715	<i>h⁺ gtb1-29</i>	This study
W716	<i>h⁻ leu1 gtb1-22</i>	This study
W721	<i>h⁻ leu1 alp4-B7</i>	This study
W722	<i>h⁺ alp4-B7</i>	This study
W725	<i>h⁻ leu1 alp4-1891 gtb1-22</i>	This study
W726	<i>h⁺ alp4-1891 gtb1-22</i>	This study
W733	<i>h⁻ leu1 alp4-1891 gtb1-93</i>	This study
W824	<i>h⁹⁰ mei1-102 gtb1-93 atb2-607</i>	This study
W865	<i>h⁺ gtb1-93 atb2-607</i>	This study
W866	<i>h⁺ gtb1-101</i>	This study
W867	<i>h⁻ leu1 gtb1-101</i>	This study
W869	<i>h⁹⁰ mei1-102 leu1 atb2-607</i>	This study
W870	<i>h⁹⁰ mei1-102 leu1 gtb1-93 atb2-607</i>	This study
W871	<i>h⁹⁰ mei1-102 leu1 gtb1-85 atb2-607</i>	This study
W872	<i>h⁹⁰ mei1-102 gtb1-22 atb2-607</i>	This study
W873	<i>h⁺ gtb1-29 atb2-607</i>	This study
W874	<i>h⁺ atb2-607</i>	This study
W875	<i>h⁺ leu1 gtb1-22 atb2-607</i>	This study
W876	<i>h⁹⁰ mei1-102 leu1 gtb1-22 atb2-607</i>	This study
W889	<i>h⁻ leu1 gtb1-85</i>	This study
W890	<i>h⁻ leu1 gtb1-29</i>	This study
W891	<i>h⁻ leu1 gtb1-93 atb2-607</i>	This study
W892	<i>h⁻ leu1 gtb1-22 atb2-607</i>	This study
W893	<i>h⁻ leu1 gtb1-29 atb2-607</i>	This study
W894	<i>h⁻ leu1 atb2-607</i>	This study
W896	<i>h⁹⁰ mei1-102 alp4⁺-3HA-kan^r</i>	This study
W897	<i>h⁹⁰ mei1-102 gtb1-93 alp4⁺-3HA-kan^r</i>	This study
YT374	<i>h⁹⁰ leu1 sid4⁺-GFP-kan^r atb2⁺-GFP-lys1⁺</i>	This study
YT375	<i>h⁹⁰ leu1 gtb1-93 sid4⁺-GFP-kan^r atb2⁺-GFP-lys1⁺</i>	This study
711-750-3D	<i>h⁻ leu1 alp4-B7 gtb1-22</i>	This study
715-750-5B	<i>h⁻ leu1 alp4-B7 gtb1-29</i>	This study

(VARDY and TODA 2000). Unlike the γ -tubulin complex in budding yeast, the γ -tubulin-Alp4/Alp6 complex in fission yeast appears to exist in larger complexes (VARDY and TODA 2000; FUJITA *et al.* 2002), analogous to the γ -tubulin complexes in higher eukaryotes.

We are interested in the function of the SPB in fission yeast, particularly during the sexual phase of the life

cycle, where the SPB, as a nuclear membrane-associated organelle, performs various tasks that are required for spindle formation, nuclear migration, spore formation, and also probably chromosome arrangement (HIRATA and SHIMODA 1994; HAGAN and YANAGIDA 1995; CHIKASHIGE *et al.* 1997; DING *et al.* 1998; NIWA *et al.* 2000). Because γ -tubulin is a key element of the SPB, we exam-

ined how the requirements of γ -tubulin are differentially regulated for the varied SPB functions in fission yeast. Here we report the isolation of several γ -tubulin mutants that suppress temperature-sensitive (*ts*) *alp4* mutations in an allele-specific manner. Sporulation, rather than vegetative proliferation, was highly susceptible to many of the γ -tubulin mutations and mutants in the components of the γ -tubulin complex differentially affected sporulation, suggesting that the function of each component of the γ -tubulin complex is differentiated at least between the vegetative and sexual phases of the life cycle in fission yeast. Also, γ -tubulin and kinesin-like proteins were functionally intimately related.

MATERIALS AND METHODS

Strains and media: Standard genetic methods for the fission yeast *S. pombe* were used (MORENO *et al.* 1991; ALFA *et al.* 1993). Yeast extract agar (YEA), malt extract agar (MEA), and Edinburgh minimal medium 2 (EMM2) were used as a complete rich medium, conjugation/sporulation medium, and minimal medium, respectively. The strains used in this study are listed in Table 1. The *alp4-1891* and *alp6-719* mutants were described by VARDY and TODA (2000). The other *ts alp4* mutants used, *alp4-B7*, *alp4-S5*, and *alp4-S7*, were created by polymerase chain reaction (PCR)-based, gene-specific mutagenesis. Briefly, a C-terminal tagging module containing green fluorescent protein (GFP; BAHLER *et al.* 1998) was fused to the *alp4*⁺ gene and PCR fragments amplified under mutagenic conditions were generated and used for substitutive transformation. The *ts* growth defect of the *alp4-B7* mutant, but not *alp4-S5* and *alp4-S7*, was rescued by a multicopy plasmid carrying the *gfb1*⁺ gene. The hemagglutinin epitope (HA) sequence was fused to the C terminus of the *alp4*⁺ gene according to BAHLER *et al.* (1998). We attempted to fuse the same sequence at the C terminus of the *alp4-1891* mutant gene that was chromosomally integrated. We also attempted to fuse the tag sequence at the N terminus in a plasmid-borne form, but failed to obtain a useful construct.

Mutant selection: Mutagenesis with nitrosoguanidine (150 μ g/ml) was performed according to UEMURA and YANAGIDA (1984). Revertants of the *alp4-1891* or *alp4-B7 ts* mutants were crossed with a wild-type strain and random spore analysis was performed. Thirty descendant colonies from each cross were tested for the *ts* phenotype and, if *ts*, the revertant was judged to carry an extragenic suppressor of the *alp4* mutant. Gene disruption using the G418-resistant marker replacement method was described previously (BAHLER *et al.* 1998). Correct disruption was verified by sequencing both of the gene replacement boundaries.

DNA sequencing: A 2.4-kb DNA fragment from 270 bp upstream of the start codon through 540 bp downstream of the termination codon of the *gfb1* gene was amplified by PCR and sequenced. Likewise, fragments covering the coding region of the *alp4* gene were sequenced.

Reagents for immunochemical analyses: A synthetic oligopeptide corresponding to the carboxyl terminus of fission yeast γ -tubulin was used to raise rabbit anti- γ -tubulin antiserum, which was affinity purified before use. Mouse monoclonal anti-HA antibody (16B12) was purchased from Constance (Berkeley, CA). Electrochemiluminescence Western blotting detection kits (Amersham, Buckinghamshire, UK) were used for Western blot analysis.

Gel-filtration chromatography: The experimental protocol described in VARDY and TODA (2000) was basically followed

for gel filtration of the γ -tubulin complexes. Briefly, cell cultures were grown in minimal medium at 28° and shifted to 36° or 20°, followed by 6 and 7 hr of incubation, respectively. Cells suspended in buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1 mM EDTA, 1 mM mercaptoethanol) containing 5 mM ATP, 1% protease inhibitor cocktail (Sigma Chemical, St. Louis), and 2 mM Pefabloc SC (Roche, Indianapolis) was disrupted with glass beads using a Multi-Beads shocker (Yasui Kikai, Osaka, Japan). Soluble cell extracts were obtained after repeated centrifugations at $18,000 \times g$ for 10 min. Immediately after the preparation, 200–250 μ l of the extract (approximate protein concentration was 30 mg/ml) was loaded on the Superose 6 10/300 GL column (Amersham), and proteins were eluted with buffer A containing 100 mM NaCl. Thyroglobulin (669 kD), apoferritin (443 kD), β -amylase (200 kD), and bovine serum albumin (66 kD; Sigma Chemical) were used as size markers. According to the suggestion of Amersham, we did not use blue dextran, a 2000-kD marker, but rather, on the basis of the specification provided by the manufacturer, we assumed the void volume to be >2000 kD.

Immunoprecipitation: Whole-cell extracts were prepared in TEG buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 15% glycerol, 1% NP-40) containing 1% protease inhibitor cocktail and 2 mM Pefabloc SC by the glass beads disruption method. The soluble extracts, each containing 2 mg of protein, were brought to the indicated concentrations of NaCl and incubated at 0° for 30 min. Approximately 10 μ g of the anti-HA antibody was added and incubated at 4° for 1 hr. Prewashed Affi-Prep Protein A support (Bio-Rad, Hercules, CA) was then added, followed by 1 hr of incubation at 4°. In some experiments (see Figure 4), the antibody was preincubated with the protein A beads at 4° for 1 hr in the absence of NaCl, mixed with the cell extracts, and incubated for 1 hr at 4° in the presence of the desired concentration of NaCl. The protein A beads were washed with TEG buffer at 4° using Spin Filters (CytoSignal, Irvine, CA) and proteins that remained bound to the beads were examined by Western blot analysis.

Cytologic analysis: Indirect immunostaining was performed with anti- α -tubulin monoclonal antibody (TAT1; WOODS *et al.* 1989) and with rabbit anti-Sad1 polyclonal antibody as described previously (GOTO *et al.* 2001; TANGE *et al.* 2002). The chromosomally integrated GFP-fused α -tubulin gene expressed under the control of the *nda2* promoter was a kind gift from H. Masuda (Kansai Advanced Research Center, Kobe, Japan). The yeast strain expressing GFP-fused Sid4 protein was used as an SPB marker (TOMLIN *et al.* 2002). The plasmid pDQ105 (DING *et al.* 1998) carrying a GFP-fused α -tubulin gene was also used to visualize microtubules. The meiosis shown in Figure 6B were induced in malt extract broth and galactose and mannose synthetic medium at 30° as described in TANGE *et al.* (1998). To examine the effect of suppressor *gfb1* mutations on cytoplasmic microtubules in interphase cells of the *alp4* mutant, synchronous cultures were made by the hydroxyurea arrest and release method as described in VARDY and TODA (2000).

Modeling of the γ -tubulin structure: A 3-D structure model of fission yeast γ -tubulin was created based upon that for the α/β -tubulin heterodimer (NOGALES *et al.* 1998) using 3D Jigsaw (Version 2) software. RASMAC software was used to graphically view the 3-D structure.

RESULTS

Determination of mutation sites in *alp4* mutant genes:

Mutation sites were determined in four different *ts alp4* mutants. One of these, *alp4-1891*, was originally isolated from a collection of fission yeast mutants that displayed

FIGURE 1.—Suppression of *alp4-1891* by *gtb1-93* mutation. Strains with the indicated mutations were plated on YEA plates and incubated at the indicated temperatures for 3 days (35° and 30°) or for 6 days (20°). HM123 (wild type), W604 (*gtb1-93*), W705 (*alp4-1891*), and W733 (*alp4-1891 gtb1-93*) were used.

altered growth polarity (VARDY and TODA 2000). The others were created by PCR-based gene-specific mutagenesis (MATERIALS AND METHODS). Among them, *alp4-B7* was suppressed by multiple copies of the *gtb1*⁺ gene, which encodes γ -tubulin in fission yeast, while *alp4-S5*, *alp4-S7*, and *alp4-1891* mutants were not suppressed. *alp4-1891* had an amino acid substitution: Ser at 194 was changed to Pro (S194P). The other mutants contained multiple substitutions: I499M, Q567K, and R619H in *alp4-B7*, P390H and L427M in *alp4-S5*, and R698Q and D735G in *alp4-S7*.

Isolation of suppressors of *ts alp4* mutants: To examine whether the *ts alp4* mutants can be suppressed by altering the γ -tubulin protein sequence, we first sought extragenic suppressors of the *alp4-1891* mutant. Mutated *alp4* mutant cells were plated and incubated at 36°, the restrictive temperature for the mutant. Of 96 colonies examined, 7 had a severe growth defect at 20°. Each of these 7 revertants was crossed with a wild-type strain and 3 of them produced *ts* progeny, indicating that they had lost the *ts* phenotype due to extragenic suppressors. Sequencing of genomic DNA indicated that one of them had a substitution mutation (P302L) in the *gtb1* gene. This mutation was identical to that

TABLE 2
 γ -Tubulin mutants isolated as suppressors
of the *alp4-1891* mutation

Allele	Mutation sites	No. of isolates
<i>gtb1-93</i>	P302L	3
<i>gtb1-85</i>	L301S	4
<i>gtb1-22</i>	P302S, V307I	1
<i>gtb1-29</i>	N87D	2

previously isolated by PALUH *et al.* (2000) as a synthetic lethal mutation with a complete loss-of-function mutation in the *pkll1*⁺ gene that encodes a member of the Kar3-family kinesin-like protein (PALUH *et al.* 2000 described that the substitution occurred at position 301, but we followed the numbering of the Sanger Center Fission Yeast Database and designated the mutation to be at 302). We named the mutant allele *gtb1-93*. Further genetic analysis by tetrad dissection confirmed that the mutation was responsible for suppression of the *alp4* mutation. Consistent with the previous result (PALUH *et al.* 2000), the *gtb1-93* mutation alone conferred a cold-sensitive (*cs*) phenotype, which was more severe than that of the *gtb1 alp4* double mutant (Figure 1, bottom). This indicated that the *alp4-1891* mutation functioned, although weakly, to suppress *gtb1-93*.

Another screening was performed to isolate more *gtb1* mutants. In the second screening, the *cs* phenotype was not taken into account. From 20 revertants due to extragenic suppressors, we isolated a new *gtb1* mutant, *gtb1-85*, which had an L301S substitution. Like *gtb1-93*, the *gtb1-85* mutant was *cs*, although the growth defect was slightly leaky. The *cs* phenotype of *gtb1-85* was partially relieved by the *alp4-1891* mutation (data not shown). Thus, two *cs gtb1* mutants and an *alp4* mutant were mutually suppressing, consistent with direct *in vivo* interactions between these two gene products (VARDY and TODA 2000).

Both the *gtb1-93* and the *gtb1-85* mutant, when crossed with themselves or with each other, produced abnormal asci containing an aberrant number and shape of spores even at high temperatures (see below). Thus, we further screened for *gtb1* mutants from revertants of *alp4-1891*, as those produced aberrant asci when crossed with the *gtb1-93* mutant. This screening revealed eight independent mutants that produced abnormal asci. All eight were verified to be *gtb1* mutants by nucleotide sequencing. As summarized in Table 2, *gtb1* mutants isolated as suppressors of *alp4-1891* were classified into four groups with respect to the mutation sites. The majority of the mutants had mutations at position 301 or 302, while the remaining *gtb1-29* carried a substitution at position 87.

Allele-specific suppression of *alp4* by the *gtb1* mutants: The clustered mutation sites in a class of suppressor *gtb1* mutants and the fact that two *cs* mutants of this class mutually suppressed the *alp4-1891* mutation

TABLE 3
Allele-specific suppression of *alp4* by *gtb1* mutants

<i>gtb1</i> allele	<i>ts alp4</i> alleles		
	<i>alp4-1891</i>	<i>alp4-B7</i>	<i>alp4-S5</i>
<i>gtb1</i> ⁺	— ^a	—	—
<i>gtb1-93</i>	++	— (lethal ^b)	— (lethal)
<i>gtb1-85</i>	++	— (lethal)	Not tested
<i>gtb1-22</i>	++	— (slightly <i>cs</i>)	—
<i>gtb1-29</i>	++	+	— (very sick)
<i>gtb1-101</i>	—	++	+/-

^a Colony-forming ability of mutant with indicated combinations of *gtb1* and *alp4* alleles at 36° on YEA plates. Colony size: ++ (large), + (medium), +/- (small), — (not visible).

^b Not viable at either 26° or 30°.

suggested that the mutated region interacts directly with Alp4 and γ -tubulin proteins. If this is the case, it might be that the suppression is specific to particular alleles of the *alp4* gene. To test this, we combined the *gtb1* mutations with different *alp4* alleles and examined their colony-forming ability (Table 3). The *gtb1-93*, *gtb1-85*, and *gtb1-22* mutants did not suppress *alp4* alleles other than *alp4-1891*. The *gtb1-93* mutation was lethal at any temperature examined (33°, 30°, and 26°) when it was combined with *alp4-B7* or *alp4-S5*. *gtb1-85* had a similar but weaker synergistic effect with these *alp4* mutants. In contrast, *gtb1-29* suppressed the *alp4-B7* and *alp4-1891* mutations. Both *gtb1-22* and -29 mutants became *cs* in the background of the *alp4-B7* mutation.

Another screening was performed for *gtb1* mutants that suppress the *alp4-B7* mutant. From eight independent batches of mutated *alp4-B7* cells, 23 revertants due to extragenic suppressors were isolated and sequenced for the *gtb1* gene. We obtained five independent mutants (six mutants in total) that had a substitution mutation in the coding region of the gene. All of them had an identical mutation: Ser at 263 was changed to Asn (named *gtb1-101*). The *gtb1-101* mutation suppressed not only the *alp4-B7* but also the *alp4-S5* mutant, although the suppression was weak for the latter mutation, while it did not suppress the *alp4-1891* mutation at all (Table 3). The *gtb1-29* allele was not isolated from the new screening, indicating that the screening was not exhaustive. Nevertheless, two different *alp4* mutants, *alp4-1891* and *alp4-B7*, were suppressed by a different range of γ -tubulin mutants. In particular, *gtb1* alleles with a substitution at 301 or 302 seemed to have very specific suppressor activity for *alp4-1891*, consistent with the idea that these sites are in a region that is involved in direct protein interactions (PALUH *et al.* 2000 and see DISCUSSION).

The *gtb1-101* mutant cells were examined to determine if they contained more γ -tubulin protein, because the *alp4-B7* mutant was suppressed by an increased dosage of the *gtb1*⁺ gene (data not shown). γ -Tubulin protein was not increased in the *gtb1-101* mutant as deter-

mined by measuring the band intensity in a Western immunoblot analysis (data not shown). The amount of γ -tubulin was also not appreciably changed in the *gtb1-93*, *gtb1-22*, or *gtb1-29* mutants.

Suppression of the *gtb1-93* mutation: We obtained a spontaneous revertant of *gtb1-93* that lost the *cs* growth defect, producing normal-sized colonies at 22°. Subsequent genetic analysis revealed that the reversion was due to a mutation in the *atb2* gene (*atb2-607*), which encoded altered α 2-tubulin with a P173L substitution. This *atb2* mutation, either alone or with *gtb1-93*, conferred hypersensitivity to the microtubule-destabilizing drug, thiabendazole (TBZ). It did not form colonies in the presence of 10 μ g/ml TBZ at 33°. The *atb2-607* mutation also suppressed another *cs* mutant, *gtb1-85*. The *cs* growth defect of the *gtb1-93* mutant is suppressed by a TBZ-sensitive allele of *nda2* (α 1-tubulin; PALUH *et al.* 2000). Thus, both α 1- and α 2-tubulin, when altered to presumed microtubule-destabilizing forms, suppressed the *cs* phenotype of the γ -tubulin mutants. Consistently, a low concentration of TBZ partially rescued the *cs* growth defect of the *gtb1* mutants (Figure 2). Figure 2 also demonstrates that the non-*cs* *gtb1* mutants, *gtb1-22*, *gtb1-29*, and *gtb1-101*, were more resistant than wild-type cells to TBZ.

Gel-filtration analysis of the γ -tubulin complex in *gtb1* mutants: We performed a gel-filtration analysis to examine whether the *gtb1-93* mutation affects the formation of the γ -tubulin complex. Protein extracts were prepared from cells with or without *gtb1-93* and *alp4-1891* mutations that were grown at 36° or at 20°. Consistent with previous reports (VARDY and TODA 2000; FUJITA *et al.* 2002), a major part of γ -tubulin in the *alp4* mutant as well as in wild-type cells was eluted near the void volume, which corresponded to >2000 kD, and this profile was not appreciably changed by the *gtb1* mutation irrespective of the growth temperature (Figure 3). When extracts of cells grown at 36° were compared, however, there was a discrete peak centering around 550 kD in the *gtb1* single mutant that was barely detectable in other strains, including the *gtb1 alp4* double mutant. Alternatively, in these strains, there was a broad distribution of γ -tubulin in fractions ranging from ~400 to 150 kD. The *alp4-ts* mutant contained a reduced amount of γ -tubulin in this range of fractions compared with wild type, while the gel-filtration pattern of the *alp4 gtb1* double mutant was very similar to that of the wild type. Thus, the effect of a single mutation of the *gtb1* and *alp4* genes on the chromatographic properties of protein complexes appeared to negate each other. This result was consistent with the mutual suppression of these mutations described above. The 550-kD peak in the *gtb1* mutant became less prominent when extract was prepared from cells grown at 20° (Figure 3), the restrictive temperature for the *gtb1* mutant, suggesting that the complex formation was somehow impaired at this temperature.

We examined whether the apparent peak shift ob-

FIGURE 2.—Effect of TBZ on *gtb1* mutants. Strains with the indicated *gtb1* allele were plated on YEA plates containing the indicated concentration (in micrograms per milliliter) of TBZ and incubated at the indicated temperatures for 3 days (30° –TBZ), 4 days (30° +TBZ and 26°), 5 days (22°), or 6 days (20°). Strains used were HM123, W604, W650, W665, W671, and W867.

served in the *gtb1-93* mutation was specific to this mutation. In both *gtb1-22* and *gtb1-29* mutants grown at 36°, there was a shift to higher-molecular-weight fractions, although the shift was not as evident as in *gtb1-93*. Between these two mutants, the *gtb1-29* mutant had a clearer shift, and interestingly, the gel-filtration pattern was affected only slightly by the *alp4-1891* mutation (data not shown).

FIGURE 3.—Gel-filtration analysis of the γ -tubulin complex. Soluble cell extracts were prepared from HM123 (wild type), W705 (*alp4-1891*), W604 (*gtb1-93*), and W733 (*alp4-1891 gtb1-93*), which were incubated at either 36° or 20°. Each fraction was analyzed by Western blotting with anti- γ -tubulin antibody. Positions of size markers (669, 443, 200, and 66 kD) were determined by a parallel control chromatography (see MATERIALS AND METHODS).

Altered stability of the γ -tubulin complex in *gtb1* mutants: Because the gel filtration was performed in the presence of 100 mM NaCl to avoid nonspecific protein interaction, the observed difference in the protein distribution could have been due to differential stability of the protein complex at various salt concentrations. Thus, we made a tagged version of the Alp4 protein in which the HA epitope sequence was fused to the C terminus of Alp4 (see MATERIALS AND METHODS). Previous studies indicated that this fused protein retained its function (VARDY and TODA 2000). Cell extracts were prepared from two strains that carried either the *gtb1*⁺ or the *gtb1-93* allele together with the *alp4*⁺-HA gene. Immunoprecipitation with an anti-HA antibody revealed that the amount of wild-type γ -tubulin that co-immunoprecipitated with Alp4-HA decreased sharply with increased concentrations of NaCl, while mutant γ -tubulin remained largely associated with Alp4-HA (Figure 4). Similar experiments were performed with other *gtb1* alleles, *gtb1-85*, *gtb1-29*, and *gtb1-22*. Gtb1-85 was stably associated with Alp4-HA, while Gtb1-29 had slightly reduced stability and Gtb1-22 had almost wild-type stability with Alp4-HA (data not shown). Thus, the salt stability of each mutant γ -tubulin appeared to be generally correlated with the chromatographic property. Therefore, the peak shift in the gel-filtration chromatography that occurred in the *gtb1* mutants might have been partially due to altered stability of the γ -tubulin complex from the salt. Although further biochemical analyses are needed to elucidate the nature of the protein complexes separated in the gel chroma-

FIGURE 4.—Immunoprecipitation analysis of Gtb1-Alp4 interaction. Cell extracts were prepared from strains W896 (wild type) and W897 (*gtb1-93*) grown at the indicated temperature and used for immunoprecipitation with anti-HA antibody. Proteins bound in the presence of the indicated concentrations of NaCl were detected with anti-HA (Alp4-HA) and anti- γ -tubulin antibodies. The precipitation of both Alp4-HA and γ -tubulin was dependent on anti-HA antibody and protein A beads (data not shown).

tography, it is probable that the *gtb1* mutation affects the stability of the γ -tubulin complex.

The salt-resistant property of the mutant γ -tubulin complex was only slightly changed by the growth temperature (Figure 4), yet the chromatographic pattern was different; that is, the prominent peak around 550 kD was apparently shifted to a lower-molecular-weight form in the extract of cells grown at a low temperature (Figure 3). Therefore, the reduction of the 550-kD peak in this extract was unlikely due to altered salt sensitivity, suggesting that the *gtb1* mutant cells grown at low temperatures contained a reduced amount of a particular form of the γ -tubulin complex.

Aberrant cytoplasmic microtubule arrays in *gtb1* mutants: Wild-type fission yeast cells in the interphase contain six to eight microtubule bundles that run mostly parallel to the long axis of the cell (HAGAN 1998). PALUH *et al.* (2000) reported that the *gtb1-93* mutant cells at a permissive temperature contained aberrant cytoplasmic microtubule bundles that were fewer in number and longer than wild-type microtubules. This observation was verified by immunofluorescent staining with an anti- α -tubulin antibody (Figure 5A). Similar aberrant cytoplasmic microtubules were observed by use of GFP-tagged α 2-tubulin (DING *et al.* 1998; Figure 5B). The suppressor *atb2* for the *cs gtb1-93* mutant negated the detrimental effect of the *gtb1* mutation on microtubule formation (Figure 5A). In *gtb1-85* and *gtb1-22* mutants, cytoplasmic microtubule arrays visualized with GFP-fused tubulin were similar to those in *gtb1-93*. *gtb1-29* mutant cells contained two types of abnormal microtubular arrays. One type contained microtubules similar to those in the *gtb1-93* mutant (Figure 5B, bottom left), but the other type had thinner microtubules (Figure 5B, bottom right). On the other hand, cytoplasmic microtubules formed in the *gtb1-101* cells were only marginally different from wild type (data not shown).

FIGURE 5.—Abnormal cytoplasmic microtubule bundles formed in *gtb1* mutants. Strains YT106 (wild type), W607 (*gtb1-93*), W650 (*gtb1-85*), W870 (*gtb1-93 atb2-607*), W871 (*gtb1-85 atb2-607*), and W869 (*atb2-607*) were used. (A) Effect of *atb2* mutation on microtubule formation. Cells with the indicated mutations were incubated at 33° in YE medium and stained with anti- α -tubulin antibody (TAT-1, green) and 4',6-diamidino-2-phenylindole (DAPI; red). (B) Interphase cells carrying the indicated *gtb1* alleles were transformed with the plasmid pDQ105 to express GFP-fused α -tubulin at 30° in EMM2 containing 10 μ M of thiamine. Bars, 5 μ m.

The *alp4* and *alp6* mutants contain aberrant cytoplasmic microtubules that resemble those in the *gtb1* mutants (VARDY and TODA 2000). We examined whether the combination of the *alp4-1891* mutation with its suppressor *gtb1-22* could reverse the aberrant cytoplasmic microtu-

FIGURE 6.—Aberrant sporulation and meiosis in the *gtb1* mutant. (A) Spores were produced by crossing HM123 and HM487 (wild type) or W604 and W609 (*gtb1-93*) on an MEA plate. Incubation was at 30° for 2 days. (B) Meioses were induced in YT374 (wild type; a, meiosis I; b, meiosis II) and YT375 (*gtb1-93*; c and d, meiosis I; f, meiosis II) at 30°. Sad1 (red), Sid4-GFP and Atb2-GFP (green), DAPI (blue). Bars, 5 μ m.

bules to the wild-type configuration. The double mutant, however, contained mutant cytoplasmic microtubules (data not shown). Similar results were obtained with the *gtb1-93* mutation. Thus, although the *gtb1* mutants suppressed lethal spindle dysfunction caused by the *alp4* mutation (data not shown), they appeared not to affect the cytoplasmic microtubules.

Impaired sporulation and meiosis in *gtb1* mutants: As mentioned above, *gtb1* mutants isolated as suppressors of the *alp4-1891* mutation produced aberrant asci containing an abnormal number and shape of spores (Figure 6A). We examined the sporulation defect quantitatively by counting the number of mature spores per ascus and compared this number with that of other *gtb1*-related mutants. The incubation temperature was either 30° or 33° where *gtb1* mutants had no or only a slight defect in vegetative growth on the basis of the colony size on a rich medium plate (Figure 2) and from the doubling time of the logarithmic phase of liquid cultures (Figure 7). Four *gtb1* mutants that suppressed the *alp4-1891* mutation produced aberrant asci and two *cs* mutants, *gtb1-93* and *gtb1-85*, were more severely impaired (Table 4). Another mutant, *gtb1-101*, which suppressed the *alp4-B7* but not the *alp4-1891* mutation, had no sporulation defect. The *gtb1-101* allele, therefore, had no discernible phenotype in either the vegetative

or the sporulation phase, except for its suppressor activity to *alp4-B7* and *alp4-S5* mutants as well as increased resistance to TBZ. We also examined the effect of *alp4-1891* and *alp6-719* mutations on sporulation. These mutants had only marginal sporulation defects at both 30° and 33°. At 33°, vegetative growth of the *alp4-1891* mutant was very poor (Figure 7). Although the *alp4-1891* mutant itself conferred little sporulation defect, it had marked synergy with the *gtb1-93* and *gtb1-22* mutants (Table 4). Note that the *alp4-1891* mutation suppressed the vegetative growth of the *cs gtb1-93* mutant, but had an adverse effect on sporulation in the same *gtb1* mutant. The other *alp4* mutant, *alp4-B7*, had defective sporulation, although the defect was not as severe as in the *gtb1* mutants. Nevertheless, the *ts* growth defect of *alp4-B7* was not as severe as that of *alp4-1891* (data not shown). These results demonstrated that mutations in the genes that encode the components of the γ -tubulin complex exhibit differential effects on vegetative growth and on sporulation. The findings also indicated that sporulation in fission yeast was generally more susceptible to *gtb1* mutation than to *alp4* and *alp6* mutations.

We further examined meiotic divisions in the *gtb1-93* mutant that occurred prior to sporulation. Two defective phenotypes were observed in meiosis I; one was impaired chromosome segregation with an apparently

FIGURE 7.—The effect of temperature on vegetative growth of *gtb1* and *alp4/6* mutants. (Left) Logarithmic phase of cultures in YE liquid medium at 26° were shifted to the indicated temperature at time 0 hr and aliquots of cultures were taken for microscopically counting the number of cells. HM123 (wild type: circle), W705 (*alp4-1891*: triangle), and W549 (*alp6-719*: square) were used. (Right) Liquid cultures at 33° were shifted to the indicated temperatures and the increase in cell number was plotted. HM123 (wild type: circle), W604 (*gtb1-93*: triangle), W715 (*gtb1-29*: square), and W716 (*gtb1-22*: diamond) were used. For all cultures, cell concentrations at time 0 were between 1.5×10^6 and 2.2×10^6 cells/ml. In the graphs, relative increase in cell number was plotted.

normal spindle (Figure 6B, c and e) and the other was a monopolar spindle (d). Figure 6B(f) shows a defective phenotype, probably in meiosis II. How these defects are related to the sporulation phenotype is not clear. Detailed analyses of the sexual phase of γ -tubulin mutants are in progress.

Microtubule-destabilizing agents rescued the sporulation defect in *gtb1* mutants: Because the *cs gtb1* mutants were suppressed by the *atb2-607* mutation and also partially suppressed by a low concentration of TBZ, both of which are presumed microtubule-destabilizing conditions, we examined whether the sporulation defect of *gtb1* mutants was affected by the same agents. The results summarized in Table 5 demonstrated the following. The *atb2-607* mutation suppressed defective sporulation in the *gtb1-93*, *gtb1-22*, and *gtb1-29* mutants. TBZ (10 μ g/ml) was as effective as the *atb2* mutant in rescuing sporulation in all of these *gtb1* mutants. Thus, aberrant sporulation occurring in *gtb1* mutants might be correlated with stabilized forms of microtubules, and thus microtubule-destabilizing agents could abrogate the deleterious effect of microtubules with altered stability. The *gtb1-101* mutant had an elevated resistance to TBZ, yet it produced almost normal asci (Table 5). The *gtb1-101* mu-

TABLE 4
Abnormal ascus formation in *gtb1* and related mutants

Crosses ^a	No. of spores per ascus			
	1	2	3	4
Wild type	0	1.1	1.1	97.8
<i>gtb1-93</i>	25.3 ^b	38.5	14.9	21.3
<i>gtb1-85</i>	11.3	30.7	20.8	37.2
<i>gtb1-22</i>	2.7	6.8	7.5	82.9
<i>gtb1-29</i>	3.0	10.5	13.9	72.6
<i>gtb1-101</i>	0	0.8	0.8	98.4
<i>alp4-1891</i>	0	1.4	0.7	97.9
<i>alp4-B7</i>	1.1	4.9	3.8	90.2
<i>alp4-1891 gtb1-93</i>	39.8	44.4	10.5	5.3
<i>alp4-1891 gtb1-22</i>	4.9	11.2	15.0	68.9
Wild type	0.0	1.9	1.9	95.9
<i>gtb1-93</i>	17.8	32.1	20.6	29.6
<i>alp4-1891 gtb1-93</i>	33.0	42.7	17.6	6.8
<i>alp4-1891</i>	0	0.8	1.9	97.3
<i>alp4-B7</i>	4.0	3.6	7.6	84.8
<i>alp6-719</i>	0	1.9	2.3	95.8

^a Strains used: HM487 and HM123 (wild type), W604 and W609 (*gtb1-93*), W714 and W889 (*gtb1-85*), W711 and W716 (*gtb1-22*), W715 and W890 (*gtb1-29*), W866 and W867 (*gtb1-101*), W705 and HM753 (*alp4-1891*), W721 and W722 (*alp4-B7*), W601 and W733 (*alp4-1891 gtb1-93*), W725 and W726 (*alp4-1891 gtb1-22*), and W549 and HM754 (*alp6-719*). Spores were produced on a MEA plate after incubating for 2 days at 30°. Incubation temperature was 33° for the bottom six crosses in this table.

^b Percentage of asci containing indicated number of spores. At least 250 asci were counted for each cross.

tant was less resistant to TBZ than the other two *gtb1* mutants, *gtb1-22* and *gtb1-29* (see Figure 2), and also did not contain abnormal cytoplasmic microtubules, like the other γ -tubulin mutants.

Genetic interactions of γ -tubulin with kinesin-like proteins: Previous studies demonstrated that a null allele of the *pk11* gene (encoding a Kar3 family of kinesin-like protein) created by a gene-disruption method (hereafter called *pk11* Δ) was synthetically lethal with the *gtb1-93* mutant (PALUH *et al.* 2000). To confirm the previous observation, we made a genetic cross to produce double-mutant strains at the *gtb1* and *pk11* loci. The strains were viable but produced smaller colonies at 33° compared to the respective single mutants. Also, the cold sensitivity of the *gtb1-93* mutant was enhanced in the *pk11* Δ background. Thus, although the *pk11*-null mutation was not synthetically lethal with the *gtb1* mutant at high temperatures, at least in the genetic background of our strains, there was a clear genetic interaction between these genes. A similar genetic interaction was reported in *Aspergillus nidulans* using a *klpA* (a homolog of *pk11*) deletion mutant and a γ -tubulin mutant (PRIGOZHINA *et al.* 2001). Another Kar3-related protein in fission yeast is encoded by *klp2*⁺ (Troxell *et al.* 2001). Unlike *pk11*,

TABLE 5
Effect of thiabendazole and the *atb2* mutation on sporulation

Crosses	TBZ concentration ($\mu\text{g/ml}$)	No. of spores per ascus			
		1	2	3	4
Wild type	0	0	0.4	1.1	98.5
	5	0.4	1.1	2.3	96.2
	10	0.4	1.5	2.9	95.3
<i>gtb1-93</i>	0	20.3	37.9	21.4	20.3
	5	5.0	18.6	14.7	61.6
	10	6.3	12.0	11.6	70.1
<i>gtb1-93 atb2-607</i>	0	5.3	15.5	10.2	68.9
<i>gtb1-22</i>	0	4.2	8.0	12.2	75.7
	10	1.1	1.9	1.5	95.5
<i>gtb1-22 atb2-607</i>	0	0	1.4	2.9	95.7
<i>gtb1-29</i>	0	4.4	11.6	11.0	73.0
	10	2.6	2.6	1.6	93.2
<i>gtb1-29 atb2-607</i>	0	0	2.7	3.1	94.2
<i>atb2-607</i>	0	0	1.0	1.7	97.2

Strains used: W865 and W891 (*gtb1-93 atb2-607*), W875 and W892 (*gtb1-22 atb2-607*), W873 and W893 (*gtb1-29 atb2-607*), and W874 and W894 (*atb2-607*). Other strains are shown in Table 4. Sporulation was induced on MEA plates containing indicated concentrations of TBZ at 30°. After incubating for 2 days, the percentage of asci containing the indicated number of spores was determined. At least 300 asci were counted for each cross.

the *klp2 Δ* mutant had very little synergy with the *gtb1* mutation in vegetative growth. This finding, consistent with the previous result (TROXELL *et al.* 2001), demonstrated that *pk11⁺* and *klp2⁺* genes have different roles in fission yeast. In the sexual life cycle, however, there was a severe defective phenotype in the *klp2 gtb1* double mutant (Y. TANGE and O. NIWA, unpublished results).

Fission yeast contains two kinesin-like proteins, encoded by the *klp5⁺* and *klp6⁺* genes, which belong to the Kin I family (WEST *et al.* 2001). This family of proteins has microtubule-destabilizing activity (DESAI *et al.* 1999). Klp5 and Klp6 share overlapping functions in both mitosis and meiosis (WEST *et al.* 2001, 2002; GARCIA *et al.* 2002a,b). The *klp5/6* mutants produce aberrant asci and abnormally elongated cytoplasmic microtubules that resemble those observed in the *gtb1* mutants in the present study (WEST *et al.* 2001; GARCIA *et al.* 2002a). Hence, we examined whether there were any genetic interactions between the *gtb1⁺* and *klp5⁺/klp6⁺* genes. The *gtb1-22* and *gtb1-29* mutants had very little synergy with either of the null mutants of the *klp5/6* genes, but combining the *gtb1-93* mutation with the *klp5* gene was lethal at any temperature examined (33°, 30°, and 26°). Similarly, cells carrying *gtb1-93* and *klp6 Δ* did not form colonies at 33°. These findings indicated an intimate functional relationship between γ -tubulin and the kinesin-family protein. Both Klp5/Klp6 and γ -tubulin might thus be required for proper microtubule dynamics in fission yeast cells. The Kin I family of proteins share essential mitotic functions with Dis1 and Alp14/Myc1 kinetochore-binding proteins (NAKASEKO

et al. 2001; GARCIA *et al.* 2002b). Consistently, the *gtb1-93 dis1* double mutant was extremely sick, producing very tiny colonies that were barely viable at 33° (data not shown). As anticipated, when a plasmid carrying the *dis1⁺* gene was introduced into the double mutant, the synergistic effect completely disappeared.

We examined whether the mutations of kinesin-related genes had a synergistic effect on *alp4* and *alp6* mutations. The temperature sensitivity of *alp4-1891* as well as of *alp6-719* was greatly enhanced in the *pk11 Δ* background, while the other null mutations, *klp2 Δ* , *klp5 Δ* , and *klp6 Δ* , did not have notable synergistic effects.

DISCUSSION

The 3-D structures of polymerized α - and β -tubulin have been determined (NOGALES *et al.* 1998). Both tubulins have basically identical structures in which a core of two β -sheets is surrounded by 12 helices. We used the structure of α -tubulin as a template for modeling the 3-D structure of γ -tubulin. The predicted structure of γ -tubulin in the form of an α/γ -tubulin heterodimer is shown in Figure 8, together with mutated amino acid residues in the γ -tubulin mutants. Figure 8 illustrates that all of the mutations occur in loops between the β -strand and the helix, which are located on the predicted surface of the γ -tubulin protein. This is consistent with the interaction of the mutated regions in γ -tubulin with other cellular factors. Of particular interest were positions 301 and 302, at which the *gtb1-22*, *gtb1-85*, and *gtb1-93* mutation sites are mapped. These γ -tubulin

FIGURE 8.—A 3-D structure model of an α/γ -tubulin heterodimer. The positions of the altered amino acid residues in γ -tubulin mutants isolated in the present study are indicated.

mutants suppressed *alp4-1891*, but not other *alp4* mutants, and two *cs* mutants among the *gtb1* mutants were partially suppressed by the *alp4-1891* mutation. The allele-specific suppression as well as the mutual suppression suggests that Gtb1 and Alp4 interact directly via the protein regions containing altered amino acids in the mutants. The results of the gel-filtration analysis (Figure 3) are consistent with mutual suppression via direct protein interaction.

Another notable feature of the suppressor γ -tubulin mutants is that, although the *ts alp4* alleles were suppressed, they produced microtubules with altered dynamics so that they were more resistant to the microtubule-destabilizing drug, TBZ, or their defective phenotype was suppressed by TBZ-sensitive α -tubulin and partially by TBZ. The effect of these microtubule-destabilizing agents was more pronounced in sexual development in the mutants. In addition, one of the *gtb1* mutants was lethal when cells lacked one of the microtubule-depolymerizing enzymes, encoded by the *klp5⁺* and *klp6⁺* genes, suggesting that microtubules formed under this condition are too stable to support cell viability. Thus, an “inappropriately stabilized microtubule” might be a common characteristic of *gtb1* mutants isolated as suppressors of the *alp4* mutants. Such “stabilized microtubules” in γ -tubulin mutants have also been reported in other organisms, but are not a general property of all γ -tubulin mutants (HENDRICKSON *et al.* 2001; JUNG *et al.*

2001). Our suppressor screenings were not exhaustive and might be biased, particularly because in the second screening we selected only those with a sporulation-defective phenotype. Therefore, it is possible that there are other types of suppressor *gtb1* mutations. Nevertheless, it seems likely that the suppression of *tsalp4* is closely related to the microtubule stability phenotype. These arguments suggest that any *gtb1* mutant could suppress the *ts alp4* mutation if the *gtb1* mutant produces microtubules with adequately altered stability for the *alp4* mutation. It is not clear how this idea can be reconciled with the notion of allele-specific suppression through the postulated direct interaction of γ -tubulin and Alp4 protein as discussed above, although these ideas are not mutually exclusive.

An intriguing finding in this study was that all of the *gtb1* mutations that suppressed the temperature sensitivity of the *alp4-1891* mutant had highly defective sporulation, while the *alp4* mutant itself did not, even at temperatures at which vegetative growth was substantially compromised. Even more striking was that the *alp4-1891* mutation was able to rescue, albeit weakly, the *cs* growth defect of *gtb1-93* and *gtb1-85* mutants in the vegetative growth phase, yet it enhanced the sporulation defect of the same *gtb1* mutants in the sexual phase. These and other results strongly suggest that the functional requirements of the γ -tubulin complex are different in the vegetative and sexual phases. Because the sporulation defect was effectively rescued by either TBZ or a TBZ-sensitive form of α -tubulin, stabilized forms of microtubules might be deleterious to sporulation. This is consistent with the fact that reduced expression of the Kin I family of kinesin-like proteins gave rise to the sporulation defect (WEST *et al.* 2001), which was very similar to that observed in the *gtb1* mutants. In the normal sporulation process, a characteristic modification of the SPB occurs during meiosis II, which is required for forespore membrane deposition and subsequent spore wall formation (HIRATA and SHIMODA 1994; IKEMOTO *et al.* 2000). The fusion of two SPBs and nuclear membranes, which occurs during conjugation prior to meiosis, is also required for normal sporulation and meiosis (TANGE *et al.* 1998). We demonstrated that at least one of the *gtb1* mutants was highly defective in meiosis. This defect is likely to be closely related to the impaired sporulation. However, detailed cytologic analyses are needed to determine the stage of meiosis and/or sporulation that is impaired in the γ -tubulin mutants and how such defects are related to the postulated altered microtubule stability. Such analyses might also elucidate further functions of γ -tubulin, particularly in the sexual development of fission yeast.

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