Functional Dissection of the \(\gamma\)-Tubulin Complex by Suppressor Analysis of \(gtb1\) and \(alp4\) Mutations in \textit{Schizosaccharomyces pombe}

Yoshie Tange,* Akiko Fujita,† Takashi Toda† and Osami Niwa*†

*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, \(\gamma\)-tubulin (encoded by the \(gbl^+\) gene), Alp4 (Spc97/GCP2), and Alp6 (Spc98/GCP3) are essential components of the \(\gamma\)-tubulin complex. We isolated \(gbl\) mutants as allele-specific suppressors of temperature-sensitive \(alp4\) mutations. Mutation sites in \(gbl\) 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 \(\gamma\)-tubulin molecule that might directly interact with the Alp4 protein. The cold sensitivity of \(\gamma\)-tubulin mutants was almost completely suppressed by an \(\alpha\)-tubulin mutation and partially suppressed by a low concentration of thiabendazole, a microtubule assembly inhibitor. Other \(gbl\) mutants had increased resistance to this drug. Gel-filtration and immunoprecipitation analyses suggested that the mutant \(\gamma\)-tubulin formed an altered \(\gamma\)-tubulin complex with increased stability compared to wild-type \(\gamma\)-tubulin. In most \(gbl\) mutants, sexual development was impaired, and aberrant ascii 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 \(\gamma\)-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 \(\gamma\)-tubulin with several kinesin-like proteins.

Ubiquitous in eukaryotes, \(\gamma\)-tubulin is a key component of the microtubule-organizing center (MTOC), such as the centrosome in animal cells. \(\gamma\)-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 \textit{Saccharomyces cerevisiae}, \(\gamma\)-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 \(\gamma\)-tubulin ring complex (\(\gamma\)-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 \(\gamma\)-TuRC. The \(\gamma\)-tubulin small complex (\(\gamma\)-TuSC) consists of Dgrip83, Dgrip91, and \(\gamma\)-tubulin and is thought to be structurally equivalent to the yeast Spc97/Spc98/\(\gamma\)-tubulin complex (Oegema et al. 1999). In vitro, \(\gamma\)-TuSC is capable of nucleating microtubules, but much less efficiently than \(\gamma\)-TuRC (Oegema et al. 1999). Genetic studies in several lower eukaryotes revealed that the nucleation of microtubules is not the sole function of \(\gamma\)-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 \(\gamma\)-tubulin protein achieves these functions and how the \(\gamma\)-tubulin complex is regulated remain to be determined.

\(\gamma\)-Tubulin in the fission yeast \textit{Schizosaccharomyces pombe} is encoded by \(gbl^+\) (also known as \(tug^1^+\)) and is essential for viability (Horio et al. 1991; Stearns et al. 1991). Fission yeast carries apparent homologs of Spc97 and Spc98 in \(S.\) \textit{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 \(\gamma\)-tubulin and localizes at the MTOC in fission yeast.
(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 Toda 2000; 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-
inhibited 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 mutants 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-S3, 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 gbl1+ 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 μg/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 gbl1 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 Convance (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°C and shifted to 36°C or 20°C, 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 shoker (Yasui Kikai, Osaka, Japan). Soluble cell extracts were obtained after repeated centrifugations at 18,000 × g for 10 min. Immediately after the preparation, 200–250 μl 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-amylose (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 2000kD 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°C for 30 min. Approximately 10 μg of the anti-HA antibody was added and incubated at 4°C for 1 hr. Prewashed Affi-Prep Protein A support (Bio-Rad, Hercules, CA) was then added, followed by 1 hr of incubation at 4°C. In some experiments (see Figure 4), the antibody was preincubated with the protein A beads at 4°C for 1 hr in the absence of NaCl, mixed with the cell extracts, and incubated for 1 hr at 4°C in the presence of the desired concentration of NaCl. The protein A beads were washed with TEG buffer at 4°C 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; TANG 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 meioses shown in Figure 6B were induced in malt extract broth and galactose and mannose synthetic medium at 30°C as described in TANG et al. (1998). To examine the effect of suppressor gbl1 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 (NOGALLES 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...
Table 2

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation sites</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtb1-93</td>
<td>P302L</td>
<td>3</td>
</tr>
<tr>
<td>gtb1-85</td>
<td>L301S</td>
<td>4</td>
</tr>
<tr>
<td>gtb1-22</td>
<td>P302S, V307I</td>
<td>1</td>
</tr>
<tr>
<td>gtb1-29</td>
<td>N87D</td>
<td>2</td>
</tr>
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Previously isolated by Paluh et al. (2000) as a synthetic lethal mutation with a complete loss-of-function mutation in the pkl1+ 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.
suppressed 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 alp4 gene. To test this, we combined the alp4 allele was not isolated from the new screening, but also the extragenic suppressors were isolated and sequenced for which corresponded to gtb1-93 mutants in total) that had a substitution mutation in the alp4 gene (data not shown). The gtb1-101 mutation affects the formation of the atb2-607, which lost the atb2 gene (attb2-607), which encoded altered atb2-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 μg/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 nota2 (α1-tubulin; Paluh et al. 2000). Thus, both α1- and α2-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 130 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 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-
Suppressor Analysis of γ-Tubulin Complex

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).

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°C 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°C in EMM2 containing 10 μM of thiamine. Bars, 5 μm.

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 α2-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).

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 microtubule arrays in these mutants.
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°C or 33°C 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°C and 33°C. At 33°C, 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
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 athb-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 athb-607 mutation suppressed defective sporulation in the gtb1-93, gtb1-22, and gtb1-29 mutants. TBZ (10 μg/ml) was as effective as the athb2 mutation 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 mutant 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 pkl1 gene (encoding a Kar3 family of kinesin-like protein) created by a gene-disruption method (hereafter called pkl1Δ) 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 pkl1 loci. The strains were viable but produced smaller colonies at 33°C compared to the respective single mutants. Also, the cold sensitivity of the gtb1-93 mutant was enhanced in the pkl1Δ background. Thus, although the pkl1-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 klp4 (a homolog of pkl1) 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 pkl1,
the klp2Δ 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 kpl1+ 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. Ntwa, 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). Klpp5 and Klpp6 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 klp6Δ did not form colonies at 33°. These findings indicated an intimate functional relationship between γ-tubulin and the kinesin-family protein. Both Klpp5/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 kpl1Δ background, while the other null mutations, klp2Δ, klp5Δ, and klp6Δ, 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

---

**TABLE 5**

Effect of thiabendazole and the atb2 mutation on sporulation

<table>
<thead>
<tr>
<th>Crosses</th>
<th>TBZ concentration (μg/ml)</th>
<th>No. of spores per ascus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>gtb1-93</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>29.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.0</td>
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<tr>
<td></td>
<td>10</td>
<td>6.3</td>
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<tr>
<td>gtb1-93 atb2-607</td>
<td></td>
<td>5.3</td>
</tr>
<tr>
<td>gtb1-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>1.1</td>
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<tr>
<td>gtb1-22 atb2-607</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>gtb1-29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>4.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.6</td>
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<tr>
<td>gtb1-29 atb2-607</td>
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<td>0</td>
</tr>
<tr>
<td>atb2-607</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

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°C. 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.
Suppressor Analysis of γ-Tubulin Complex

![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.](image)

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.

We thank Aki Minoda (Cancer Research UK) for modeling of the protein 3-D structure and Jan Paluh for discussion. We also thank Hirohisa Masuda (Kansai Advanced Research Center) and Kathy Gould (Vanderbilt University) for supplying the yeast strains, and...


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