Identification of Novel Temperature-sensitive Lethal Alleles in Essential β-Tubulin and Nonessential α2-Tubulin Genes as Fission Yeast Polarity Mutants

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We have screened for temperature-sensitive (ts) fission yeast mutants with altered polarity (alp1–15). Genetic analysis indicates that alp2 is allelic to atb2 (one of two α-tubulin genes) and alp12 to nda3 (the single β-tubulin gene). atb2+ is nonessential, and the ts atb2 mutations we have isolated are dominant as expected. We sequenced two alleles of ts atb2 and one allele of ts nda3. In the ts atb2 mutants, the mutated residues (G246D and C356Y) are found at the longitudinal interface between α/β-heterodimers, whereas in ts nda3 the mutated residue (Y422H) is situated in the domain located on the outer surface of the microtubule. The ts nda3 mutant is highly sensitive to altered gene dosage of atb2+; overexpression of atb2+ lowers the restrictive temperature, and, conversely, deletion rescues ts. Phenotypic analysis shows that contrary to undergoing mitotic arrest with high viability via the spindle assembly checkpoint as expected, ts nda3 mutants execute cytokinesis and septation and lose viability. Therefore, it appears that the ts nda3 mutant becomes temperature lethal because of irreversible progression through the cell cycle in the absence of activating the spindle assembly checkpoint pathway.

INTRODUCTION

Microtubules are important and ubiquitous structures that play essential roles in various cellular processes, including motility, mitosis, transport of proteins and mRNAs, and cell morphogenesis (reviewed in Mitchison and Kirschner, 1986; Lehmann, 1995; Hyman and Karsenti, 1996). They assemble from heterodimers composed of α- and β-tubulin subunits, which are both evolutionarily highly conserved. Additional members of tubulin subfamilies including γ-tubulin exist in various species (Oakley, 1992; Burns, 1995). The crystallographic structure of the α/β-heterodimer has been solved recently (Nogales et al., 1998); however, many fundamental aspects of microtubule function and dynamics are still not fully understood.

Genetically amenable organisms such as yeasts and fungi have proven to be ideal systems with which to investigate the cellular function of microtubules (Oakley and Morris, 1981; Neff et al., 1983; Hiraoka et al., 1984; Toda et al., 1984; Schatz et al., 1986a). Saccharomyces cerevisiae (budding yeast) and Schizosaccharomyces pombe (fission yeast), although very divergent in evolution, have analogous genomic organization of tubulin-encoding genes (i.e., two α-tubulin genes and one β-tubulin gene) and show similar genetic properties in those genes (Neff et al., 1983; Hiraoka et al., 1984; Toda et al., 1984; Adachi et al., 1986; Schatz et al., 1986a,b). In both yeasts, a single β-tubulin-encoding gene is essential for cell viability (nda3+ in fission yeast and TUB2 in budding yeast) (Neff et al., 1983; Hiraoka et al., 1984). In contrast, two homologous α-tubulin-encoding genes (nda2+ and atb2+ in fission yeast, and TUB1 and TUB3 in budding yeast) show different genetic behavior. One of the two homologues is essential for cell viability (nda2+ and TUB1), whereas the other (atb2+ and TUB3) is not; however, the nonessential α-tubulin can compensate for loss of the essential α-tubulin gene when overexpressed, indicating that these two pairs of α-tubulin molecules

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are functionally interchangeable (Adachi et al., 1986; Schatz et al., 1986b).

Determining the molecular basis for the temporal and spatial definition of cell polarity is a fundamental problem in cell biology. The shape of every eukaryotic cell is believed to be maintained by the cytoskeleton, which consists of three filamentous systems: actin-based microfilaments, tubulin-based microtubules, and intermediate filaments. The cytoskeleton does not act as a static structural scaffold of the cell. Instead, in virtually every system characterized to date, the cytoskeleton is highly dynamic, frequently changing its structure during cell cycle progression and developmental differentiation. This dynamic behavior is intimately related to growth control (Drubin and Nelson, 1996; Görczy and Hyman, 1996).

Fission yeast cells are rod shaped and have defined growth polarity during both the vegetative cycle and developmental states (Mitchison and Nurse, 1985; Snell and Nurse, 1993). Previous work from our laboratory and other laboratories has clearly shown the importance of microtubule integrity in determining growth polarity. Mutations in the tubulin genes (Toda et al., 1983; Umesono et al., 1983b) or their regulators (Mata and Nurse, 1997; Hirata et al., 1998) result in altered cell shapes such as bent or branched cells. The cortical actin-dependent pathway is also important for the maintenance of growth polarity as well as cytokinesis (Gould and Simanis, 1997). As in other organisms, the localization of these cytoskeletal molecules changes dramatically during cell cycle progression (Marks and Hyams, 1985; Marks et al., 1986; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988), and these molecules play key roles in specific cell cycle events, as demonstrated by the fact that genes encoding cytoskeletal components and regulators were first identified as cell division cycle (cdc) mutants (Nurse et al., 1976; Gould and Simanis, 1997).

To understand in more detail the molecular pathways that regulate cell polarity, we have undertaken a large-scale screen of temperature-sensitive (ts) mutants to identify those with altered growth polarity (alp) (Hirata et al., 1998). We have isolated a class of alp mutants that become bent or branched and lose microtubules after incubation at the restrictive temperature. Genetic as well as molecular analyses indicate that two of these alp loci are allelic to atb2 and nda3, encoding a2-tubulin and β-tubulin, respectively. The identification of mutations in the atb2 gene as ts polarity mutants is intriguing because previous studies have shown that cells in which atb2 is deleted are viable, with few defects (Adachi et al., 1986), which suggests that the alp2 mutants we have isolated here must be dominant in nature. The identification of nda3 as a ts mutant is also interesting because despite extensive and systematic mutational analysis of the β-tubulin gene in the past, very few ts mutants have been isolated (Yamamoto, 1980; Oakley and Morris, 1981; Umesono et al., 1983a,b; Thomas et al., 1985; Huffaker et al., 1988; Matsuzaki et al., 1988; Stearns and Botstein, 1988; Reijo et al., 1994; Sage et al., 1995). We have determined, therefore, the mutation sites of the tubulin genes in these ts mutants. There is a single point mutation in each mutant, affecting codons that correspond to amino acids that are highly conserved through evolution. Furthermore, in contrast to previously isolated tubulin mutants, detailed phenotypic analyses suggest that the spindle assembly checkpoint control might not be operational in these ts tubulin mutants.

**MATERIALS AND METHODS**

**Strains, Media, and Chemicals**

All mutant strains were derived from HM123 (h leu1) (Table 1). JY6 (h leu1his2), and TP108-3D (h leu1 thr4his2) were used for backcrossing mutants. Complete medium, YPD (1% yeast extract, 2% polypeptone, and 2% dextrose), which contains 10 μg/ml Phloxine B (Sigma, St. Louis, MO) (called YPDP), YES (0.5% yeast extract, 3% dextrose, and 75 μg/ml adenine, histidine, leucine, and uracil), modified synthetic EMM2, and MES (3% malt extract, and 75 μg/ml adenine, histidine, leucine, and uracil) have been described previously (Moreno et al., 1991). Plates contained 1.6% agar.

**Genetic Techniques and Nomenclatures**

Standard procedures for S. pombe genetics were followed as described (Moreno et al., 1991). Cell number was measured using Sysmex F-800 (TOA Medical Electronics, Tokyo, Japan). S. pombe cells were transformed using the lithium method (Ito et al., 1983). A temperature-sensitive phenotype is abbreviated to the lowercase letters ts, e.g., ts atb2. Proteins are designated by an uppercase first letter, e.g., Atb2. Gene disruptions are abbreviated as the gene preceded by Δ such as Δatb2.

**Isolation of ts Mutants with Polarity Defects**

Wild-type HM123 cells were mutagenized with N-methyl-N’-nitro-N-nitrosoguanidine as described previously (Uemura and Yanagida, 1984). Approximately 300 viable cells were spread per one YPDP plate. Plates were incubated at 29°C for 4 d, and colonies were then replica plated on YPDP and incubated at 36°C for 1 d. Strains that did not form colonies or that formed sick dark-red colonies were picked, and the morphology of these cells was examined by Calcofluor staining (Streiblová and Wolf, 1972). Approximately 1 of 50 colonies showed either no growth or retarded growth at the restrictive temperature. In total, 200,960 colonies were screened in this way, and 2822 ts mutants were isolated. Mutants that showed altered cell shape (bent or branched) at 36°C were selected for further analysis. These mutants were grown in liquid YPD and shifted to 36°C for 8 h, and the cell morphology was examined once again by Calcofluor staining. In total, 22 ts mutants that showed bent, curved, or branched morphologies were isolated and designated alp (Table 2).

**Complementation Analysis**

h+ mutant strains were isolated after mating of the mutant strains to JY6 (h leu1his2). Crisscross mating was performed, and free spores were plated on two YPDP plates, one of which was incubated at 29°C, the other at 36°C. If the difference in the number of colonies formed between these two plates was >10-fold, the two mutants were assigned as allelic. Strain 1146 (alp8) (Table 2) contained two
mutations: one is responsible for the morphological defects, which is allelic to \textit{toa1} (Mata and Nurse, 1997), and the other is responsible for the ts growth phenotype.

**Dominance–Recessive Test**

A stable prototrophic diploid heterozygous for the \textit{atb2} locus was constructed between the ts \textit{mei1-B102atb2-996} strain (TPR19A) (Table 1) and a mating strain (TP71-7C), and temperature sensitivity was examined.

**DNA Preparation and Manipulation**

Standard molecular biology techniques were followed as described (Sambrook et al., 1989). Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA, and Boehringer Mannheim, Indianapolis, IN). Nucleotide sequencing was performed by the dideoxy method (Sanger et al., 1977).

**Identification of the \textit{alp2} and \textit{alp12} Loci as \textit{atb2} and \textit{nda3}, Respectively**

\textit{S. pombe} genomic libraries constructed in the vectors pDB248 (Beach and Nurse, 1981; Hirano et al., 1988) and pUR19 (Barbet et al., 1992) were used for the isolation of plasmids that complemented the ts \textit{alp2} or \textit{alp12} mutant (DH1-7C; \textit{h} \textit{leu1} \textit{alp2} [\textit{atb2}]-996; DH1-2D; \textit{h} \textit{leu1} \textit{ura3} \textit{alp2} [\textit{atb2}]-996; and PR7: \textit{h} \textit{leu1} \textit{ura3} \textit{alp12} [\textit{nda3}]-1828) (Table 1). For the \textit{atb2} mutant, 4 of 36,000 colonies transformed with the pDB248-based library were capable of growing at 36°C, whereas for \textit{alp2}, 5 of 20,000 colonies transformed with the pUR19-based library grew at 36°C. Segregation analysis indicated that the \textit{Ts} \textit{atb2} phenotype was plasmid dependent. Plasmid DNAs were recovered from these transformants. Four different plasmids (pALA200, pALB200, pALC200, and pALD200) and three different plasmids (pCR8 [isolated three times], pCR9, and pCR10) were recovered from the \textit{alp2} and \textit{alp12} transformants, respectively. Restriction mapping indicated that three (pALA200, pALC200, and pALD200) contained overlapping inserts and carried the \textit{atb2} gene, whereas pALB200 contained the \textit{nda3} gene. pCR8, 9, and 10 all contained overlapping inserts and carried the \textit{nda3} gene.

Identification of the \textit{alp2} loci as \textit{atb2} has been described previously (Yaffe et al., 1996). Furthermore, tetrad analysis between \textit{ts atb2-996} and an \textit{atb2} disruptant (\textit{atb2}) (Table 1) showed a tight linkage; 23 tetrads were dissected, and all showed parental ditypes. Allelism between \textit{alp2} and \textit{nda3} was determined as follows. pCR10 was transformed into the ts \textit{alp2} (\textit{nda3})-1828 mutant. At 36°C, a number of \textit{Ts} \textit{Ura} colonies appeared that stained less intensely on YPDP (at an approximate frequency of \textit{10}^{-3}). Segregation analysis indicated that the \textit{Ura} phenotype was stable, showing that pCR10 had integrated stably in the genome via homologous recombination. Free spore analysis between these integrants and a mating strain (TP108-3D) (Table 1) showed that no ts segregant appeared from 10\textsuperscript{5} colonies, indicating that \textit{alp2} is allelic to \textit{nda3}.

**Cloning of the ts \textit{atb2} and \textit{nda3} Genes and Determination of the Mutation Sites**

To determine the mutation sites in the ts \textit{atb2} mutants, 0.6-kb (corresponding to the first 166 residues of \textit{Atb2}) and 1.3-kb (residues 167–449) HindIII fragments that comprise the entire \textit{atb2} gene (Toda et al., 1984) were cloned into a vector and used to transform ts \textit{atb2-996} and -1212 strains (Yaffe et al., 1996). Both of the ts mutants were suppressed by the plasmid containing the 1.3-kb fragment but not by that containing the 0.6-kb fragment, indicating that the mutation sites of \textit{atb2} and -1212 are located in the 1.3-kb HindIII fragment. A DNA fragment corresponding to amino acids 167–449 was amplified from ts \textit{atb2-996} and -1212 strains by PCR using the following oligonucleotides as primers: Alp2-N1, Alp2-C1, and Alp2-C2.

For the \textit{ts atb2} and \textit{nda3} a 1.3-kb HindIII fragment was sequenced, and the nucleotide sequences were determined. Comparison of the nucleotide sequences of the \textit{atb2} and \textit{nda3} genes revealed that the published data contain sequencing errors in eight positions. In \textit{ts nda3-1828} nucleotide 1566 (A of initiator methionine is denoted as +1) was mutated from T to C, which results in substitution of Tyr by His at residue 422 (see RESULTS). These sequence data are available from EMBL/GenBank/DDJB under accession numbers AF042827 (\textit{nda3}*) and AF042828 (\textit{nda3}–1828).

**Immunchemical Assays**

For indirect immunofluorescence microscopy, the methanol fixation method was used (Alfa et al., 1993). TAT-1 antibody (provided by Dr. Keith Gull, University of Manchester, Manchester, United Kingdom) and Cy3-conjugated sheep anti-mouse immunoglobulin G (Sigma) were used to visualize microtubules, and DAPI was used for chromosomal DNA.

Cell extracts were prepared as described (Matsusaka et al., 1995), except that HB buffer (Moreno et al., 1991) was used in the disruption of cells. Standard procedures for immunoblotting were followed (Harlow and Lane, 1988). Monoclonal anti-\textit{b}-tubulin antibody (Sigma), anti-\textit{a}-tubulin (TAT-1, gift from Dr. Keith Gull), and anti-Cdc2 (Y100, gift from Dr. Hiroyuki Yamano, ICRF) were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad, Richmond, CA) and a chemiluminescence system (ECL, Amersham, Arlington Heights, IL) were used to detect bound antibody.

**RESULTS**

**Isolation of Mutants That Are Defective in Growth Polarity**

We performed a large-scale visual screen for mutants with defects in growth polarity. We first isolated ts mutants and then examined the cell morphology of these mutants after incubation at the restrictive temperature using Calcofluor, which stains septa and growing ends of the cell (Streiblová and Wolf, 1972; Mitchison and Nurse, 1985). Mutants with bent, curved, or branched morphology were selected. In total, 22 different mutants were obtained, and complementation tests indicated that these represent 15 loci, designated \textit{atb1}–15 (Table 2). Some examples of the cell morphology and DAPI staining of \textit{alp2} and 12 mutants are shown in Figure 1.
Classification of Polarity Mutants with Microtubule Staining

It has become clear that microtubules are important for the determination of cell shape and for growth polarity of fission yeast (Toda et al., 1983; Yaffe et al., 1996; Mata and Nurse, 1997; Hirata et al., 1998). We therefore used indirect immunofluorescence microscopy by using anti-tubulin antibody (TAT-1, kindly provided by Dr. Keith Gull) to examine the microtubule cytoskeleton of the alp mutants. It was clear from these analyses that at least four (alp1, 2, 11, and 12) of the genes identified in our screen were required for the maintenance of microtubule structures, because the microtubules in these mutants became fragile and disappeared when the temperature shifted. In this study, we have characterized alp2 and alp12 at the molecular level. Three alleles of alp2 (-996, -1212, -1377) (Table 2) and one allele of alp12 (-1828) were identified. Microtubule staining of alp2 and alp12 mutants is shown in Figure 2. Cytoplasmic microtubules became unstable and disappeared after 6 h incubation at 35.5°C, although some short nuclear spindle microtubules still remained (Figure 2, B and C, bottom panels).

In addition to the loss of microtubule structures, alp2 and alp12 mutants showed various defective phenotypes in cell cycle and cell shape control. These include displacement of the nucleus (24% for alp2-996 and 17% for alp12-1828 after 8 h at 35.5°C) (Figure 1) and accumulation of septated cells. Also, asymmetrical rather than medial septation was often observed in many cells (~50% of septated cells), resulting in anucleate cells following cytokinesis (8% for alp2-996 and 2% for alp12-1828).

Identification of the alp2 and alp12 Mutations as Novel Alleles in Mutant Tubulin Genes

We cloned the alp2 and alp12 genes by complementation using a fission yeast genomic library constructed with a multicopy vector. Four plasmids were isolated that suppressed ts alp2-996, and three suppressed alp12-1828. Restriction mapping of these plasmids indicated that the four plasmids suppressing the

<table>
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<tr>
<th>Loci</th>
<th>Alleles</th>
<th>Morphology*</th>
<th>Microtubules</th>
<th>Genes/product</th>
</tr>
</thead>
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<td>No</td>
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<tr>
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<tr>
<td>3</td>
<td>149, 601</td>
<td>Curved/cut</td>
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</tr>
<tr>
<td>4</td>
<td>225, 1891</td>
<td>Bent/cut</td>
<td>Few</td>
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</table>

* Mutants that showed >1% of cells with branched morphology or 10% of “cut” at 36°C for 8 h are indicated as “branched” or “cut” (Hirano et al., 1986), respectively.

* Hirata et al., 1998.

* The original isolate (1146) contained two mutations; one in tea1 (Mata and Nurse, 1997), which leads to branched morphology at 36°C, and the other in an unknown locus, which causes temperature-sensitive growth.

* Radcliffe and Toda, unpublished observations.

Table 1. Strain list

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<tr>
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<th>Genotypes</th>
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<td>Hirata et al. (1998)</td>
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<td>macl-B102 leu1 arg1 atb2-996</td>
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Table 2. Complementation groups and defective phenotypes of the alp mutants

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alp2 mutation consist of two previously identified genes, nda2+ and atb2+, which encode the two α-tubulins (α1- and α2-tubulin, respectively) (Toda et al., 1984; Adachi et al., 1986), and all three alp12-complementing plasmids contained the nda3+ gene, which encodes β-tubulin (Hiraoka et al., 1984). Genetic analysis indicated that alp2 was allelic to atb2, and alp12 was allelic to nda3 (see MATERIALS AND METHODS). Therefore, we shall hereafter use atb2 for alp2 and nda3 for alp12 preceded by ts, such as ts atb2.

Mutations in tubulin genes, or genes involved in microtubular pathways, often result in supersensitivity to antimicrotubule drugs such as members of the benzimidazole family of compounds (Umesono et al., 1983b; Adachi et al., 1986; Hirata et al., 1998). Consistent with this, ts atb2 and nda3 strains were supersensitive to the benzimidazole compound thiabendazole. They could not form colonies on rich media plates containing 10 μg/ml at 20°C, whereas wild-type cells could (Table 3).

Genetic Interaction of ts atb2 and nda3 Mutations with Other Tubulin Genes

It was curious that the nonessential atb2 gene was identified in this study as a ts mutation. The mutant Atb2 proteins (α2-tubulin) must have a dominantly harmful effect on the maintenance of microtubules because deletion of atb2+ does not show a lethal or ts phenotype (Adachi et al., 1986). In line with this notion, genetic analysis demonstrated that the ts atb2 mutant is dominant because a heterozygous diploid between atb2-996 and atb2+ showed the ts phenotype, although some weak growth was observed compared with a haploid ts alp2-996 strain (Figure 3A). If the mutant Atb2 protein were to inhibit the function of β-tubulin by abortive binding, effectively taking the β-tubulin out of the pool of available subunits, it might be expected that an increased dosage of β-tubulin gene would suppress ts atb2 mutations; however, a multicopy plasmid containing nda3+ was incapable of suppressing ts atb2 (Figure 3B, top right plate). This result suggests that the phenotypic dominance of ts atb2 (α2-tubulin) over nda2+ (α1-tubulin) is not ascribable to a loss of β-tubulin function.

Crossing ts atb2-996 and cs nda2-KM52 (Umesono et al., 1983b) indicates that the double mutants are synthetically lethal. Among 12 tetrads dissected, no double mutants (14 spores predicted) were obtained, whereas 14 wild-type and 10 of each single mutant strain formed colonies at 29°C, a temperature permissive for both mutants. Thus for both ts atb2 and cs nda2 mutants, functional wild-type proteins of the other α-tubulin homologue are required for viability at the permissive temperature.

In the case of ts nda3, a different effect from increased dosage of α-tubulin-encoding genes was observed. Overexpression of atb2+ led to enhanced temperature sensitivity in the ts nda3 mutant (Figure 3B, bottom left plate). Incubation of ts nda3 on minimal plates at 34.5°C allowed colony formation in the ts nda3 mutant containing an empty vector, whereas mutant cells containing a multicopy plasmid carrying atb2+ were incapable of forming colonies at this temperature. This suggested that the ts nda3 mutant is highly sensitive to altered gene dosage of atb2+. To examine the effect of atb2+ gene dosage in more detail, the atb2+ gene was disrupted in the ts nda3 strain. In reverse parallel with the toxic effect of overexpression, the deletion of atb2+ partially rescued the temperature sensitivity of nda3-1828 (Figure 3C). This result indicates that the defective phenotypes observed in the ts nda3 mutant can be ascribed, at least in part, to the existence of
the Atb2 protein. It is noteworthy that the toxic effect is α2-tubulin specific, because ts nda3 cells carrying the nda2+ gene encoding α1-tubulin on an equivalent plasmid were capable of forming colonies, although of a slightly smaller size compared with those containing vector alone (Figure 3B, bottom left plate). Multicopy plasmids containing γ-tubulin (Horio et al., 1991) do not suppress either ts atb2 or nda3, nor do they enhance the lethality conferred by these mutations (Hirata and Toda, unpublished observations), suggesting that the interactions we report are specific.

Determination of Mutation Sites in the ts atb2 and nda3 Mutants
We determined the mutation sites of the ts atb2 and nda3 mutants (atb2-996 and -1212 and nda3-1828; see MATERIALS AND METHODS). In each case, a single point mutation was found, which resulted in alteration of an amino acid residue in Atb2 and Nda3. In ts atb2-996, nucleotide 1065, guanine (A of the initiator methionine is denoted as +1), was mutated to adenine, which resulted in the substitution of cysteine 356 (TGT) with tyrosine (TAT; mutated nucleotides are underlined). In the case of ts atb2-1212, nucleotide 737,
guanine, was mutated to adenine, which resulted in the substitution of glycine 246 (GGC) with aspartate (GAC). A comparison of amino acid sequences around the mutated residues is shown in Figure 4A. Both of the mutated residues have been highly conserved throughout evolution. In particular, glycine 246 is of interest because this residue is invariant not only in fission yeast α1-tubulin (Nda2) and α- tubulins from other species, but also in β- and γ-tubulins in virtually every species (Figure 4A).

The mutation site of ts nda3-1828 is located near the C terminus of the Nda3 protein. Nucleotide 1566 was mutated from thymine to cytosine, which results in the substitution of tyrosine 422 (TAT) with histidine (CAT) (Figure 4B). Like glycine 246, this tyrosine is also conserved in all members of tubulins. It is noteworthy that the region around tyrosine 422 is rich in acidic amino acid residues, which are believed to be important for interactions with other proteins. Extensive mutational analysis of the budding yeast β-tubulin gene TUB2 has shown the C terminus of β-tubulin to be essential for the normal function of microtubules (Reijo et al., 1994).

**ts atb2 Alters the Cellular Ratio of α1- to α2- Tubulin**

Why do the atb2 mutations that we isolated show a dominant ts growth defect? As shown above, it is not due to the absorption of β-tubulin. One possible explanation might be an altered cellular ratio of α1- and α2-tubulins. To examine this possibility, immunoblotting using anti-α-tubulin antibody was performed with protein extracts from a ts atb2 mutant. As described previously (Adachi et al., 1986), the two closely migrating bands of 55 and 57 kDa in wild-type extracts correspond to Atb2 and Nda2, respectively (Figure 5A, lane 1) (note that p57

<table>
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<th>Strainsa</th>
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<td>−</td>
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<tr>
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<td>++</td>
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<tr>
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Table 3. Sensitivity of the atb2 and nda3 mutants to thiabendazole (TBZ)

Strains used are as follows: ts atb2 (DH1-7C; see Table 1); ts nda3 (DH12); alp1 (DH2-8D; Hirata et al., 1998); and Δatb2 (atb2::LEU2; Adachi et al., 1986); cs nda2 (h- leu1nda2-KM52; Toda et al., 1984); and wild type (HM123).

b Mutant cells were streaked on rich YPD plates containing 10 μg/ml of TBZ and incubated at 28° or 20°C for 7 d. + +, Colony size was indistinguishable from that on plates without TBZ: +, tiny colonies were formed. −, no colonies were formed.

c NA, Not applicable.

The mutation site of ts nda3-1828 is located near the C terminus of the Nda3 protein. Nucleotide 1566 was mutated from thymine to cytosine, which results in the substitution of tyrosine 422 (TAT) with histidine (CAT) (Figure 4B). Like glycine 246, this tyrosine is also conserved in all members of tubulins. It is noteworthy that the region around tyrosine 422 is rich in acidic amino acid residues, which are believed to be important for interactions with other proteins. Extensive mutational analysis of the budding yeast β-tubulin gene TUB2 has shown the C terminus of β-tubulin to be essential for the normal function of microtubules (Reijo et al., 1994).

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mislocalized septa (Figure 6B). Also, nuclear displacement was evident, and in some cells partial chromosome segregation occurred (Figure 1B). Polarity defects such as bent or branched cell morphology were apparent at 2 h. Consistent with continued cell cycle progression in these mutants, interphase-like hemisphere chromosomes, rather than condensed chromosomes, were seen after 6 h at 35.5°C (Figure 1B). This contrasts with the cs allele of nda3 (-KM311), which results in accumulation of increasing numbers of cells with condensed chromosomes with prolonged incubation at the restrictive temperature (Umesono et al., 1983a; Hiraoka et al., 1984).

To examine the reversibility of the ts nda3 mutant incubated at the restrictive temperature, the mutant cells were incubated for various periods (2, 4, and 6 h) at 35.5°C before being returned to 26°C, and the percentage of mitotic or septated cells was measured. No synchronous anaphase or septation was observed (Figure 6C). These results show that contrary to the cell cycle arrest phenotype of cs nda3 mutants, in the ts nda3 mutant the spindle assembly checkpoint is not functional, and mutant cells proceed into mitosis and subsequent events such as septation and cytokinesis at the restrictive temperature. A similar result was obtained with a ts atb2 mutant, namely mitosis, and septation occurred at the restrictive temperature, although viability remained high (Figure 1A) (Radcliffe and Toda, unpublished observations).

If the ts nda3 mutant becomes lethal because of cell cycle progression in the absence of activation of the spindle checkpoint, it would be expected that the lethality of the mutant would be rescued by blockage of the cell cycle before entry into mitosis. That is indeed the case. The ts nda3 mutant arrested in early S phase by hydroxyurea (HU) treatment did not lose viability for up to 4 h incubation at the restrictive temperature, whereas the control culture in the absence of the drug lost viability sharply (Figure 6D). Further incubation at the restrictive temperature in the presence of HU (>4 h) led to a loss of viability of the ts nda3 mutant, probably because of progression of the cell cycle by prolonged exposure to HU (21% after 6 h) (Sazer and Nurse, 1994). We therefore conclude that the loss of viability in the ts nda3 mutant is ascribable to irreversible progression of the cell cycle without normal function of spindles, in which the spindle assembly checkpoint is not activated.

**DISCUSSION**

**Cell Polarity and Microtubules**

Microtubules have been shown to be indispensable in the execution of diverse cellular processes in many systems, including motility, mitosis, protein and mRNA transport, and cell morphogenesis. In fission
Figure 4. Determination of the mutation sites in the ts atb2 (alp2) and nda3 (alp12) mutants. Amino acid comparisons of α-tubulin (A) and β-tubulin (B) together with the amino acid substitution caused by a point mutation in each ts mutant are shown. Sp stands for fission yeast (S. pombe), Sc for budding yeast (S. cerevisiae), and Hs for human (H. sapiens). atb2-996 contains a single base change at nucleotide number 1065 (G to A, A of the initiator methionine is denoted as +1), resulting in an amino acid substitution of cysteine 356 with tyrosine. atb2-1212 contains a single base change at nucleotide 737 (G to A), which results in an amino acid substitution of glycine 246 with aspartate. nda3-1828 contains a single base change at position 1566 (from T to C), resulting in substitution of tyrosine 422 with histidine. Only nonconserved amino acid residues are shown in B for Hs and Sc. Asterisks show amino acids where the previously published data (Hiraoka et al., 1984) are incorrect because of sequencing errors.
yeast, microtubules also execute several distinct functions, including chromosome segregation (Umesono et al., 1983a, b), distribution of organelles, in particular mitochondria and Golgi (Ayscough et al., 1993; Yaffe et al., 1996), and cell polarity and morphogenesis (Toda et al., 1983; Mata and Nurse, 1997; Hirata et al., 1998). Fission yeast tubulin mutants were originally isolated on the basis of cell-cycle defective phenotypes in mitosis (nuclear division arrest [nda]) (Toda et al., 1983). In this study we have screened for ts mutants with altered polarity of cell growth (alp mutant) and have identified new mutant alleles in tubulin genes. This clearly demonstrates that, as in other eukaryotes, microtubules are crucial elements in the determination of cell polarity in fission yeast. How do microtubules regulate cell polarity? A recent study has identified a “marker” molecule that translocates into the cell tips via microtubules and determines the orientation of cell tip growth (Mata and Nurse, 1997). There may be several molecules that mark the growing tips in a microtubule-dependent manner, and other alp+ genes may encode such molecules.

Work from budding yeast, on the other hand, suggests that in this organism the main role of microtubules is in mitosis and meiosis, namely nuclear migra-

Figure 5. The level of tubulin proteins in ts atb2 and nda3 mutants. (A) Total cell extracts were prepared from wild type (HM123, lane 1; Table 1), deleted atb2 (Δatb2, lane 2), or ts atb2-1212 (1212, lanes 3–6). Cells were cultured at 26°C in lanes 1 and 2. Atb2-1212 cells were first grown at 26°C (lane 3) and shifted to 35.5°C. Samples were collected at 4 (lane 4), 6 (lane 5), and 8 (lane 6) h. Protein (2 μg) from cell extracts was run in SDS-PAGE, and immunoblotting was performed with anti-α-tubulin antibody (TAT-1). (B) ts nda3-1828 cells (DH12) were grown as described in A, and samples were taken every 2 h after the shift. Cell extracts (20 μg) were run in SDS-PAGE, and immunoblotting was performed with mouse anti-β-tubulin antibody (Sigma) (top) or anti-Cdc2 antibody as a loading control (bottom).

Why Do the atb2 Mutants That Are Isolated Become Temperature Sensitive?

How does ts α2-tubulin (Atb2) protein act in a dominant manner over α1-tubulin (Nda2) at the restrictive temperature? There are several possibilities. The first is that ts Atb2 protein forms an abortive complex with some other protein(s), which usually execute a function that is essential for microtubule biogenesis and cell viability (Figure 7A). This scenario is not unique: a similar mutant has been reported and analyzed previously. Fission yeast contains two genes encoding type I protein phosphatases, dis2+ and sds21+ (Ohkura et al., 1989). Unlike nda2+ and atb2+, neither dis2+ nor sds21+ is essential by itself, but simultaneous disruption leads to lethality. The dis2-11 mutation, however, shows a dominant cs phenotype. In this case, the reason that dis2-11 shows cs appears to be due to the absorption, by cs Dis2, of a protein called Sds22, which is a regulatory subunit of both type I protein phosphatases and is essential for cell viability (Ohkura and Yanagida, 1991; Stone et al., 1993). Multicopy plasmids containing dis2+, sds21+, or sds22+ genes are capable of suppressing cs dis2-11. These interactions are strikingly similar to the interactions between the tubulin genes that we present here. The observation that in the ts atb2 mutant the relative ratio of Nda2 and ts Atb2 protein alters in a way that ts atb2 cells appear to contain more ts Atb2 protein is intriguing. It has been reported that free α-tubulin molecules become highly unstable (Tian et al., 1997). It is possible that in the ts atb2 mutant interaction between Nda2 and other essential proteins is compromised, which may lead to an apparent reduction of the relative ratio of Nda2 and ts Atb2 protein. The rescue of ts atb2 by high-dosage nda2+ gene supports this assumption.

β-Tubulin is a strong candidate for a protein that is absorbed by the ts Atb2 protein. The data, however, show that this is probably not the case, because an increased dosage of the β-tubulin gene did not sup-
press the ts atb2 mutant. Other candidates for interacting proteins are the cofactors that are required for the correct folding of tubulin molecules (Tian et al., 1996). Cofactors B, D, and E are proposed to interact with α-tubulin to produce assembly-competent α/β-tubulin heterodimers (Tian et al., 1997). All of these molecules are essential for cell viability in fission yeast (cofactor D/Alp1 [Hirata et al., 1998; Radcliffe and Toda, unpublished observations]). Preliminary analysis from our laboratory, however, makes this possibility also unlikely because none of the fission yeast homologues of these cofactors suppress the ts atb2 mutant when introduced on multicopy plasmids (Radcliffe and Toda, unpublished observations). There may, of course, be other α-tubulin binding proteins, such as microtubule-associated proteins (MAPs), that await identification.

**The Implications of the Mutation Sites in ts Atb2 Protein**

The second possibility is that ts Atb2 protein dominantly interferes with the assembly of newly synthesized α/β-tubulin heterodimers (Figure 7A). The electron crystallographic structure of the α/β heterodimer was recently solved (Nogales et al., 1998). The tubulin molecule is divided into three functional domains, namely the N-terminal domain (1–205), which is responsible for GTP binding, the intermediate domain (206–381), which is required for heterodimer and/or intradimer formation, and the remaining C-terminal domain, which is thought to be important for interactions with various MAPs and motors. Assignment of the mutation sites of ts atb2 (-996 and -1212), which reside in the central domain, to the three-dimensional structure of α/β-heterodimers has proved illuminat-
ing. Cysteine 356 (mutated to tyrosine in atb2-996) is located at the longitudinal interface between the α- and β-tubulin monomers (Nogales et al., 1998). It is also the residue whose analogous position in β-tubulin is the binding site for the tubulin-depolymerizing drug colchicine (Bai et al., 1996). Interestingly, glycine 246 (mutated to aspartate in atb2-1212) is spatially adjacent to the β-sheet in which cysteine 356 is included, and furthermore, both of these amino acid residues are in close contact with the GTP/GDP exchangeable site of β-tubulin (Nogales et al., 1998). It is also noteworthy that the region adjacent to glycine 246 (242LRFEG246) shows homology to regions that possess ribose-binding activity within various ATPases (Burns and Farrell, 1996). All of these facts raise the interesting possibility that the ts atb2 mutation alters the physical interaction between α- and β-tubulin, or between α/β-heterodimers, and is accompanied by an alteration in the level of GTP/GDP exchange or hydrolysis of β-tubulin.

In the worm Caenorhabditis elegans, the mutation analogous to G246D was isolated in β-tubulin—encoding mec-7 (G244S, called u129) (Savage et al., 1994). Consistent with the fission yeast atb2-1212 mutant, the u129 mutant exhibits a dominant phenotype. Thus it appears that for either α- or β-tubulin, this conserved glycine has an essential role in microtubule biogenesis and/or assembly, and that mutation of this residue results in a dominant phenotype. Given the high degree of conservation in both sequence (invariant glycine) and function (dominant mutations) in higher eukaryotic systems in which mutants are not readily available, inducible expression of this mutant form of tubulin might be a useful approach for conditionally disrupting microtubules. This hypothesis is currently being tested.

**ts Mutation in the β-Tubulin-encoding nda3⁺ Gene**

The toxicity of the high-dosage atb2⁺ gene and suppression by its deletion in the ts nda3 mutant suggest an altered affinity of ts Nda3 toward the Atb2 protein. It is possible that heterodimers of Atb2/ts Nda3 might specifically interfere with microtubule assembly and/or maintenance in this mutant. However, because nda2⁺ is an essential gene, we are unable to similarly test whether its deletion rescues ts of nda3-1828. Further analysis will be required to establish any distinction between the two α-tubulin molecules in terms of their specific interaction with ts Nda3.

The mutation site in nda3-1828 resides in the C-terminal region of the molecule Y422H. In animal cells, the C-terminal region is responsible for interactions with MAPs (MAP-1 and MAP-2) (Rivas et al., 1988; Cross et al., 1991), and the crystallographic analysis shows that the C-terminal region resides on the outer surface of the microtubule (Nogales et al., 1998).

Systematic mutational analysis of budding yeast β-tubulin also suggests that regions near the C terminus are essential for microtubular function (Reijo et al., 1994), and a truncation from glutamate 431 results in a ts growth defective phenotype (Matsuzaki et al., 1988). Recently an essential protein that shows properties similar to mammalian MAPs has been identified in budding yeast (Irminger-Finger et al., 1996). It is there-
fore possible that the C-terminal region is also required for an interaction with MAPs in fission yeast and that this interaction is perturbed in the ts nda3 mutant.

Despite extensive mutational analysis of β-tubulin genes, only a few ts mutants have been successfully isolated to date. In contrast, many cs mutations have been isolated (Oakley and Morris, 1981; Thomas et al., 1985; Huffaker et al., 1988; Matsuizaki et al., 1988; Stearns and Botstein, 1988; Davis et al., 1994; Reijo et al., 1994; Savage et al., 1994; Sage et al., 1995). In view of this, the nda3-1828 mutant is of interest and may be a useful tool with which to obtain further insight into the structure and function of microtubules.

Mitotic Spindle Assembly Checkpoint and ts Tubulin Mutants

A surprising observation arising from this study is that in both ts abt2 and nda3 mutants, it appears that the spindle assembly checkpoint is not operational (Figure 7B). In these mutants, cell cycle events such as seption, which usually occur after bipolar spindle function, continue to take place at the restrictive temperature despite defects in mitosis. In contrast, previously identified cs nda3-KM311 mutant cells arrested uniformly in midmitosis with condensed chromosomes and no septa (Umesono et al., 1983b; Hiraoka et al., 1984). This is the typical terminal phenotype when the spindle assembly checkpoint is functional. One possible explanation of this phenotypic difference is that in the ts nda3 mutant, microtubular function, especially spindle function, is insufficiently defective to activate the spindle assembly checkpoint. As shown in Figure 2, we sometimes observe short residual spindle microtubules in ts nda3 cells incubated at the restrictive temperature. A similar phenotype has been observed in ts abt2 and cs nda2 mutants (Umesono et al., 1983b; this study). Because fission yeast has two α-tubulin–encoding genes, a single mutation may be unable to completely abrogate the function of the other gene, and as a result partial microtubular function remains. It is therefore possible that the temperature lethality of nda3-1828 mutant cells arises from abortive cell cycle progression attributable to residual spindle function, preventing activation of the checkpoint machinery rather than cell cycle arrest caused by the loss of microtubules.

It is worth pointing out that the phenotype of the ts nda3 mutant, which loses viability at the restrictive temperature, is similar to that of spindle assembly checkpoint mutants such as cs nda3-KM311 mad2, nda3-KM311cdc16, and nda3-KM311dna1 double mutants in which components of the spindle assembly checkpoints are missing (Fankhauser et al., 1993; Murone and Simanis, 1996; He et al., 1997). The nda3-1828 mutant could prove useful in the genetic dissection of the transduction mechanisms that monitor spindle defects via the checkpoint machinery.

ACKNOWLEDGMENTS

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