Journal of Cell Science 110, 623-633 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS8065

The role of γ-tubulin in mitotic spindle formation and cell cycle progression in *Aspergillus nidulans*

Mary Ann Martin[1,*], Stephen A. Osmani[2] and Berl R. Oakley[1,2,†]

[1]Department of Molecular Genetics, Ohio State University, 484 W. 12th Avenue, Columbus, OH 43210, USA
[2]Weis Center for Research, Geisinger Clinic, 100 N. Academy Avenue, Danville, PA 17822, USA

*Present address: Department of Biology, 336 Jordan Hall, Indiana University, Bloomington, IN 47405, USA
†Author for correspondence at address 1

SUMMARY

γ-Tubulin has been hypothesized to be essential for the nucleation of the assembly of mitotic spindle microtubules, but some recent results suggest that this may not be the case. To clarify the role of γ-tubulin in microtubule assembly and cell-cycle progression, we have developed a novel variation of the gene disruption/heterokaryon rescue technique of *Aspergillus nidulans*. We have used temperature-sensitive cell-cycle mutations to synchronize germlings carrying a γ-tubulin disruption and observe the phenotypes caused by the disruption in the first cell cycle after germination. Our results indicate that γ-tubulin is absolutely required for the assembly of mitotic spindle microtubules, a finding that supports the hypothesis that γ-tubulin is involved in spindle microtubule nucleation. In the absence of functional γ-tubulin, nuclei are blocked with condensed chromosomes for about the length of one cell cycle before chromatin decondenses without nuclear division. Our results indicate that γ-tubulin is not essential for progression from G_1 to G_2, for entry into mitosis nor for spindle pole body replication. It is also not required for reactivity of spindle pole bodies with the MPM-2 antibody which recognizes a phosphoepitope important to mitotic spindle formation. Finally, it does not appear to be absolutely required for cytoplasmic microtubule assembly but may play a role in the formation of normal cytoplasmic microtubule arrays.

Key words: γ-Tubulin, Microtubule organizing center, Mitosis, Cell cycle, Microtubule

INTRODUCTION

Microtubule organizing centers (MTOCs) are structurally diverse organelles that are critical to the correct positioning of microtubules, regulation of microtubule assembly, and establishment of microtubule polarity (reviewed by Brinkley, 1985; Kimble and Kuriyama, 1992; Kalt and Schliwa, 1993; Rose et al., 1993). One protein that is critical to MTOC function is γ-tubulin.

A variety of experiments have indicated that γ-tubulin is important for the nucleation of microtubule assembly by MTOCs (Oakley et al., 1990; Stearns et al., 1991; Horio et al., 1991; Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994; Masuda and Shibata, 1996). These and other data have led to the suggestion that rings of γ-tubulin might nucleate the assembly of microtubules (Oakley et al., 1990; Oakley, 1992) and several observations strongly support this model. In vitro translated γ-tubulin binds to the minus ends of microtubules with a stoichiometry consistent with this model (Li and Joshi, 1995). γ-Tubulin ring complexes purified from *Xenopus* eggs nucleate microtubule assembly and cap the minus ends of microtubules (Zheng et al., 1995). Immunoelectron microscopic tomography has demonstrated, moreover, that γ-tubulin is located in ring structures in the pericentriolar material of isolated *Drosophila* centrosomes (animal MTOCs) and when microtubules assemble from the centrosomes, the γ-tubulin complexes are at the minus ends (Moritz et al., 1995).

Recent findings, however, while confirming the importance of γ-tubulin for the organization of a functional spindle, call the role of γ-tubulin in microtubule nucleation into question. If γ-tubulin rings nucleate the assembly of spindle microtubules, the elimination of functional γ-tubulin should prevent the assembly of spindle microtubules. Sunkel et al. (1995) have examined neuroblasts in *Drosophila* larvae homozygous for a P-element insertion within the 5′ untranslated region of a γ-tubulin gene. They found that mitotic spindles were structurally abnormal and the number of microtubules was reduced relative to wild-type spindles. Spindle microtubules were not eliminated, however, and this suggests that γ-tubulin may not be required for assembly of spindle microtubules but may play some other role in spindle formation.

In *Saccharomyces cerevisiae*, the sequencing of the genome has revealed a gene, *TUB4*, that encodes a protein (Tub4p) that is related to γ-tubulin (Burns, 1995; Sobel and Snyder, 1995). This protein is unusually divergent from other γ-tubulins, exhibiting only 29-38% identity to other members of the γ-tubulin family (Sobel and Snyder, 1995) and was originally designated ε-tubulin (Burns, 1995). Like γ-tubulins, however, Tub4p localizes to SPBs and is essential for viability (Sobel and Snyder, 1995). Depletion of Tub4p, or conditional

mutations in *TUB4*, perturb spindle structure but do not prevent assembly of spindle microtubules (Sobel and Snyder, 1995; Spang et al., 1996; Marschall et al., 1996). If Tub4p is functionally equivalent to the γ-tubulins of other organisms, these results, again, argue that γ-tubulin may not be required for the nucleation of the assembly of spindle microtubules.

The most persuasive genetic evidence that γ-tubulin is required for the assembly of spindle microtubules has come from *A. nidulans* (Oakley et al., 1990) where disruption of the γ-tubulin gene caused a virtually complete absence of mitotic spindles. It has been pointed out, however (Marschall et al., 1996) that in these experiments and similar experiments carried out with *S. pombe* (Horio et al., 1991), γ-tubulin loss occurred relatively long before it was possible to observe the phenotype produced by the loss. This raises the possibility that some of the phenotypes observed are secondary rather than primary consequences of γ-tubulin loss. With respect to this possibility, some discussion of the heterokaryon rescue technique that was used to determine the phenotype of a γ-tubulin gene disruption in *A. nidulans* (Oakley et al., 1990) is in order. While this technique is extraordinarily useful because it allows one to determine the phenotype of a lethal gene disruption, the phenotype can only be observed in germinating asexual spores (conidia). If conidia from a γ-tubulin disruption heterokaryon are germinated in selective medium, germlings carrying the disruption can easily be identified because they form long germ tubes while conidia carrying parental nuclei barely germinate. Since the first cell cycle normally occurs before germination, however, it is not possible to distinguish parental from γ-tubulin disruptant germlings at the point at which the first mitosis would normally occur because neither have germ tubes. It is thus possible that some of the phenotypes observed previously might not be direct effects of the absence of γ-tubulin but, rather, secondary effects caused by aberrant progression through multiple cell cycles in the absence of nuclear division (i.e. secondary consequences of the absence of γ-tubulin). In view of the importance of the data from *A. nidulans* to the hypothesis that γ-tubulin serves as a template for the assembly of spindle microtubules, it would be of great value to be able to examine γ-tubulin disruptant germlings in the first cell cycle after germination.

It would also be valuable to know if γ-tubulin has any function in the cell cycle other than a likely role in the assembly of the mitotic apparatus. In this regard, it is worth pointing out that disruption or mutation of γ-tubulin genes has not resulted in a significant elevation of the chromosome mitotic index (CMI, the percentage of cells with condensed chromosomes) (Oakley et al., 1990; Horio et al., 1991; Sunkel et al., 1995). This is surprising because, when spindle formation is blocked with antimicrotubule agents, nuclei are generally blocked in mitosis for an extended period and a greatly elevated chromosome mitotic index results. One possible explanation is that, in the absence of γ-tubulin, the transition from mitosis to G_1 occurs at a more or less normal rate even though no spindles form. If true, this would indicate that γ-tubulin is a component of an M to G_1 checkpoint and that in the absence of γ-tubulin, this checkpoint is not in place. It is important to note, however, that these experiments are all subject to the caveats expressed in the preceeding paragraph. γ-Tubulin loss or depletion occured relatively long before the observation point and the failure of nuclei to block in mitosis could be a secondary effect. In *S. cerevisiae* it is possible to examine the effects of temperature sensitive (ts^-) mutations in the *TUB4* gene on cell cycle progression. Such mutations result in several abnormal spindle morphologies at restrictive temperatures but, perhaps surprisingly, do not result in an arrest at a single point in the cell cycle (Spang et al., 1996). (The CMI cannot be determined for *S. cerevisiae* because chromosomal condensation is not evident in this organism.)

Many additional important questions about the functions of γ-tubulin remain partially or completely unanswered. It would be worthwhile, for example, to clarify the in vivo relationship between γ-tubulin and the phosphoepitopes recognized by the MPM-2 antibody (Davis et al., 1983). MPM-2 recognizes epitopes that are particularly abundant during mitosis (Vandré et al., 1984). Production of the phosphoepitopes recognized by MPM-2 correlates with increased microtubule nucleation capacity (Verde et al., 1990) and is important for assembly of mitotic microtubules from centrosomes (Centonze and Borisy, 1990) and spindle pole bodies (SPBs; fungal MTOCs) (Masuda et al., 1992). Indeed, the apparent importance of γ-tubulin and of MPM-2 reactive epitopes to microtubule assembly from MTOCs might suggest that phosphorylation of γ-tubulin is responsible for the generation of the MPM-2 reactive epitopes at MTOCs. It would also be worthwhile to know if γ-tubulin is required for SPB replication in *A. nidulans*.

We have developed a novel extension of the *A. nidulans* heterokaryon rescue technique that allows us to synchronize germlings carrying a γ-tubulin disruption and to observe the effects of the absence of functional γ-tubulin in the first cell cycle after germination. We have created γ-tubulin disruption heterokaryons in strains carrying the ts^- mutations *nim*A5 or *nim*T23. *nim*A encodes the NIMA protein kinase that is required for the G_2 to M transition (Oakley and Morris, 1983; Osmani et al., 1987, 1988b, 1991) and *nim*T encodes a phosphatase similar to that encoded by the *cdc25* gene of *Schizosaccharomyces pombe* (O'Connell et al., 1992). Each mutation blocks the cell cycle in G_2 at high temperatures and each is rapidly reversible, allowing the resumption of the cell cycle upon shifting to a lower, permissive, temperature. We have used this approach to examine the role of γ-tubulin in mitotic spindle formation, cell cycle progression, cytoplasmic microtubule assembly, SPB replication and the presence of MPM-2 reactive antigens at the SPB.

MATERIALS AND METHODS

Strains and media

We used strains G191 (*pyr*G89, *paba*A1, *fw*Al, *ua*Y9) (from Dr G. Turner, University of Bristol via Dr C. F. Roberts, University of Leicester), SO6 (*nim*A5, *pyr*G89, *w*A2, *cnx*E16, *cho*A1, *s*C12, *y*A2, *cha*A1) and SO26 (*nim*T23, *pyr*G89, *w*A2, *bi*A1, *paba*A1, *s*C12) and heterokaryon H1 (*mip*A^+ and *mip*Ad1 in a G191 background) (Oakley et al. 1990). Selective media for strains carrying *pyr*G89 were YG (5 g/l yeast extract, 20 g/l dextrose), YAG (YG with 15 g/l agar) or FYG [YG with 25 g/l Pretested Burtonite 44c (TIC Gums)]. Nonselective liquid medium for strains carrying *pyr*G89 was YG supplemented with 10 mM uridine. Nonselective solid media were YAG or FYG supplemented with 10 mM uridine and 1.0 mg/ml uracil.

Construction of *mip*A gene disruptions in ts^- cell-cycle mutant strains

We disrupted the *mip*A gene in *nim*A5 (SO6) and *nim*T23 (SO26)

backgrounds by transforming with plasmid pLO12 (Oakley et al., 1990) using the transformation procedure of Osmani et al. (1987). When pLO12 integrates into the chromosomal *mip*A gene by homologous recombination it creates a disrupted *mip*A allele, *mip*Ad1 (Oakley et al., 1990). We isolated heterokaryons and identified, by Southern hybridizations, heterokaryon H9 which carries *mip*A^+ nuclei and *mip*Ad1 nuclei in strain SO6 and heterokaryon H7 which carries *mip*A^+ nuclei and *mip*Ad1 nuclei in strain SO26.

Determination of mitotic indices and nuclear number

Conidia from control strains (SO6, SO26 and G191) were inoculated into nonselective liquid medium at a density of 1×10^6/ml and conidia from heterokaryons were inoculated at a density of 5×10^6/ml into selective medium. They were incubated, with shaking, in a New Brunswick gyrotory water bath shaker. Restrictive temperature was 42-43°C and permissive temperatures were 32°C or 25°C. Samples were fixed in 1.0% glutaraldehyde (Electron Microscopy Sciences) in the culture medium for 10 minutes. They were washed twice for 10 minutes in double distilled water and stained with mithramycin (Sigma) (Oakley and Morris, 1980) or 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma) (Oakley and Rinehart, 1985). They were mounted in the antifade compound, Citifluor AF 1 (Marivac, Ltd).

Immunofluorescence microscopy

Sterile glass coverslips were placed on the bottom of 100 mm Petri dishes containing 20 ml of liquid medium. Conidia from control strains (SO6, SO26 and G191) were inoculated at a density of 2.5×10^5/ml in nonselective medium and conidia from heterokaryons were inoculated at a density of 5×10^5/ml in selective medium. The Petri dishes were placed in styrofoam floats in a waterbath for incubation. During incubation the conidia settled onto the coverslips and adhered tightly. We used 42-43°C as a restrictive temperature and 32°C or 25°C as permissive temperatures. Procedures for immunofluorescence microscopy were modified from Oakley et al. (1990). Details of the modifications are available from the authors upon request.

We used the following primary antibodies: YOL1/34, a rat monoclonal anti-α-tubulin antibody (Accurate); TU27B, a mouse monoclonal anti-β-tubulin antibody (a generous gift from Dr L. Binder, Northwestern University School of Medicine, Chicago, Illinois via Dr G. Lozano, M. D. Anderson Cancer Center, Houston, Texas); MPM-2, a mouse monoclonal antibody against phosphoepitopes abundant in mitosis (Davis et al., 1983); a mouse monoclonal antibody against histone H1 (Leinco) and an affinity-purified rabbit polyclonal antibody against *A. nidulans* γ-tubulin (Oakley et al., 1990). Secondary antibodies were from Jackson ImmunoResearch, Inc. They were Cy3-conjugated goat anti-rat IgG, biotin-conjugated goat anti-rabbit IgG, biotin-conjugated goat anti-mouse IgG, Cy3-conjugated donkey anti-mouse IgG and Cy3-conjugated goat anti-mouse IgG. Secondary antibodies were preadsorbed by the manufacturer against serum proteins from relevant species to minimize cross reactivity in double labelling experiments.

RESULTS

Our experimental design is shown in Fig. 1. *A. nidulans* conidia are arrested in G_0 and, thus, begin the cell cycle in G_1. Upon germination the germ tube grows, nuclear divisions take place synchronously and the nuclei move to maintain an even spacing along the germ tube. (Each germling is a syncitium at this stage.) When a conidium carrying either of the ts⁻ mutations *nim*A5 or *nim*T23 is germinated at a restrictive temperature (Fig. 1b), the germ tube grows, but the nucleus is arrested in late G_2 of the first cell cycle (Oakley and Morris, 1983; Bergen et al., 1984; Osmani et al., 1987; O'Connell et al., 1992). When germlings are shifted to a permissive temperature, the G_2 block is released, and their nuclei enter mitosis and divide. By disrupting the γ-tubulin gene in these ts⁻ backgrounds (Fig. 1c) we hoped to determine what happens during the first cell cycle when functional γ-tubulin is absent.

Disruption of the *mip*A gene in synchronizable strains

Since the γ-tubulin gene (*mip*A) is essential, we used the heterokaryon rescue technique to obtain conidia carrying a γ-tubulin disruption and *nim*A or *nim*T. This technique has been described in detail previously (Osmani et al., 1988a; Oakley et al., 1990). Briefly, γ-tubulin disruption heterokaryons were created by transforming strains that carry *pyr*G89 (a mutant allele of the orotidine-5′-phosphate decarboxylase gene) with plasmid pLO12 (Oakley et al., 1990). pLO12 carries the wild-type *Neurospora crassa pyr4* gene

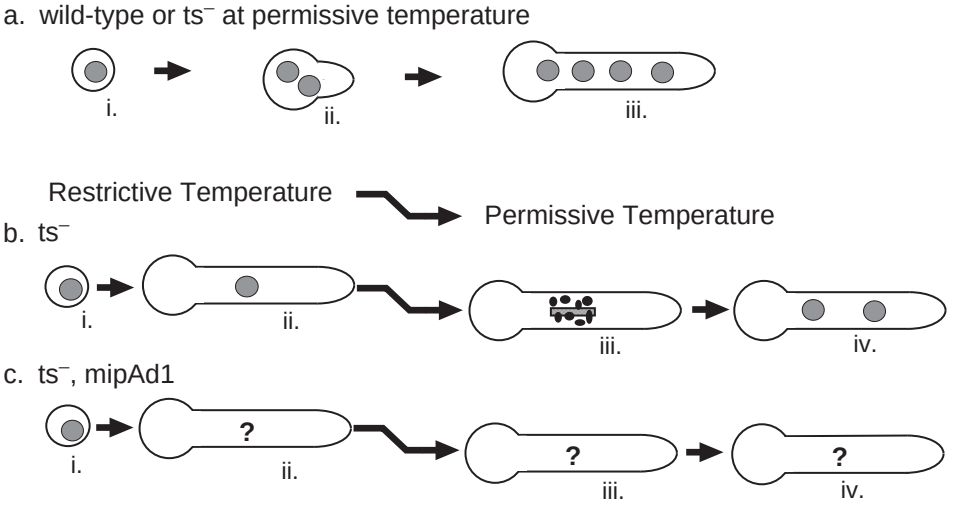

Fig. 1. Experimental design. (a) Growth of wild-type strains or ts⁻ (strains carrying *nim*A5 or *nim*T23) at a permissive temperature. (i) The single nucleus in the conidium is in G_0. (ii) The conidium swells, the nucleus then divides at about the point when the germ tube begins to extend. (iii) Additional rounds of nuclear division occur as the germ tube continues to extend. (b) *nim*A5 or *nim*T23 (ts⁻) strains germinated at a restrictive temperature and shifted to a permissive temperature. The conidium (i) germinates and extends a germ tube (ii) at the restrictive temperature but the nucleus is blocked in G_2 and nuclear division does not occur. Upon shifting to a permissive temperature, the nucleus enters mitosis (iii, chromosomes and spindle are illustrated schematically) and divides (iv). (c) Strains carrying *nim*A5 or *nim*T23 and the γ-tubulin disruption, *mip*Ad1 (ts⁻, *mip*Ad1 strains). We asked if, in the absence of functional γ-tubulin, nuclei are blocked in G_2 at the restrictive temperature, enter mitosis upon a shift to a permissive temperature and complete mitosis normally.

(*pyr4*$^+$) and a 1.1 kb internal fragment of the *mip*A gene. *pyr4*$^+$ complements *pyr*G89 and allows growth on medium unsupplemented with uridine or uracil (selective medium). Integration of pLO12 at the *mip*A locus creates the *mip*Ad1 allele that consists of two nonfunctional, truncated copies of *mip*A flanking *pyr4*$^+$. *mip*Ad1 is a recessive lethal allele so transformants carrying it are not viable. However, during transformation heterokaryons form that contain both transformed nuclei (that carry *mip*Ad1) and untransformed (parental) nuclei. In such heterokaryons, parental nuclei encode functional γ-tubulin, transformed nuclei encode the *pyr4* gene product, and the heterokaryon hyphae grow at a wild-type rate on selective medium. The conidia produced by the heterokaryon are uninucleate and have either parental (*mip*A$^+$, *pyr*G89) nuclei or transformed, disruptant (*mip*Ad1, *pyr*G89) nuclei. *mip*A$^+$, *pyr*G89 conidia have functional γ-tubulin, but require uridine or uracil and do not germinate on selective medium. *mip*Ad1, *pyr*G89, conidia carry *pyr4*$^+$ (within *mip*Ad1) and are able to germinate on selective medium but lack functional γ-tubulin.

From transformants of SO26, we isolated heterokaryon H7 which contains *mip*Ad1, *nim*T23 (and parental) nuclei and from transformants of SO6 we isolated hetrokaryon H9 (*mip*Ad1, *nim*A5 and parental nuclei). As with the original γ-tubulin disruption heterokaryon, H1 (Oakley et al., 1990), *mip*Ad1 was completely recessive in H7 and H9. We confirmed, additionally, that *mip*Ad1 is recessive by transforming protoplasts from H1 with a plasmid carrying *mip*A$^+$. The plasmid carrying *mip*A$^+$ complemented *mip*Ad1 allowing normal growth (results not shown). Finally, as will be discussed below, we verified that γ-tubulin was reduced to a level undetectable by immunofluorescence microscopy at the SPBs of germlings carrying *mip*Ad1.

We first wished to determine if, as expected, the γ-tubulin disruptant germlings from H7 and H9 gave the same phenotype at permissive temperatures as those from heterokaryon H1 (Oakley et al., 1990). DAPI-stained disruptant germlings from H7 and H9 were indistinguishable from each other and from H1. As reported for H1 (Oakley et al., 1990), the great majority of the germlings each contained a single nucleus that was generally larger than those in wild-type germlings.

We next determined the percentage of germlings in which nuclei had condensed chromosomes (chromosome mitotic index or CMI). The CMIs of the *nim*A5, *mip*Ad1 germlings from H7 (4.6±2.5%) and the *nim*T23, *mip*Ad1 germlings from H9 (4.7±2.0%) were similar to the CMI for *mip*Ad1 germlings from H1 (5.6±1.0%) and to the CMI previously reported for *mip*Ad1 germlings from H1 (5.8%) (Oakley et al., 1990). We conclude that at permissive temperatures, the phenotypes of γ-tubulin disruption germlings from H7 and H9 are identical to the phenotypes of those from H1.

Effects of a γ-tubulin gene disruption on cell cycle progression and mitotic spindle formation

In the following experiments, we germinated conidia from H7 and/or H9 in selective medium to allow germlings carrying *mip*Ad1 to be distinguished easily. As controls we germinated SO6 and/or SO26 in medium supplemented with uridine (non-selective medium). For brevity, we will refer to germlings carrying *nim*T23 or *nim*A5 and a wild-type γ-tubulin gene as ts$^-$, *mip*A$^+$ germlings. We will refer to germlings carrying *nim*T23 or *nim*A5 and a disrupted γ-tubulin gene as ts$^-$, *mip*Ad1 germlings.

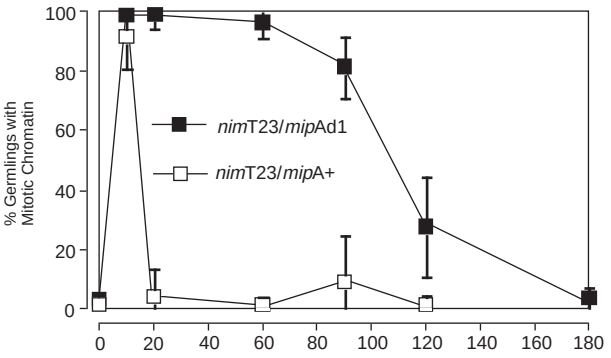

Fig. 2. CMIs of synchronized ts$^-$, *mip*A$^+$ and ts$^-$, *mip*Ad1 strains. Results with SO26 (*nim*T23, *mip*A$^+$) and H9 (*nim*T23, *mip*Ad1) germlings are shown. Similar results were obtained with strains carrying *nim*A5. Cultures were germinated at a restrictive temperature and then shifted to a permissive temperature. Samples were taken at the restrictive temperature (0 minute time point) and at intervals after the shift to the permissive temperature. CMIs (% of germlings with mitotic chromatin) were determined as discussed in Materials and Methods. Each point represents the average of three separate experiments with 350 germlings scored for each strain at each time point in each experiment. Error bars mark 95% confidence limits and in instances in which error bars are not shown, 95% confidence limits fell within the boxes used to denote the values.

We first determined the effects of the γ-tubulin disruption on entry into and exit from mitosis. We incubated H7, H9 and control conidia at a restrictive temperature for 9.5 hours and then shifted them to a permissive temperature. Samples were taken just prior to the shift to permissive temperature (0 minute time point) and at intervals following the shift. Results were similar for strains carrying *nim*A5 and *nimT23* and results for strains carrying *nim*T23 are shown in Fig. 2. As expected, the control ts$^-$, *mip*A$^+$ germlings had low CMIs at the 0 minute time point because they were blocked in G_2. Both control strains entered mitosis rapidly after the shift to permissive temperature and exhibited elevated CMIs. As mitosis was completed, nuclei entered interphase and the CMIs dropped to near 0 by 20 minutes after the shift.

Like the control strains, the ts$^-$, *mip*Ad1 germlings had low CMIs at the restrictive temperature. They also entered mitosis rapidly after the shift to permissive temperature and exhibited high CMIs. In striking contrast to the control strains, however, the γ-tubulin disruptant germlings remained blocked in mitosis for about the length of one cell cycle. The exit from the mitotic block was asynchronous and apparently gradual because many nuclei at the 90 and 120 minute time points had partially decondensed chromatin. By three hours after the shift to permissive temperature, however, the vast majority of the ts$^-$, *mip*Ad1 germlings had interphase chromatin.

In these same experiments, we examined the effects of the γ-tubulin disruption on nuclear division by determining the number of nuclei per germling. Most ts$^-$, *mip*A$^+$ germlings and all ts$^-$, *mip*Ad1 germlings had one nucleus at the G_2 block point. By 20 minutes after the shift to the permissive temperature, more than 90% of ts$^-$, *mip*A$^+$ germlings had completed mitosis and possessed two nuclei (results from three experi-

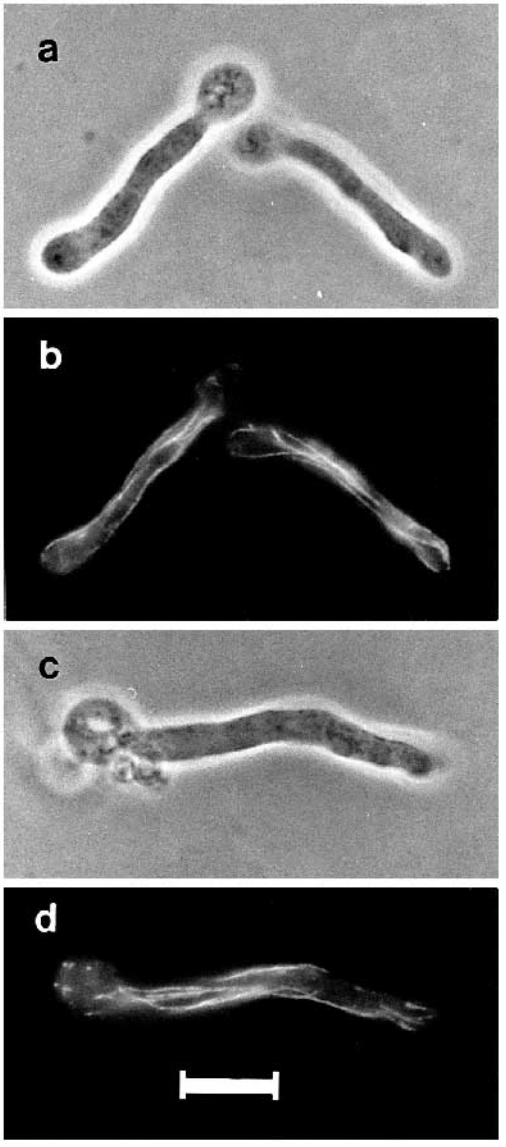

Fig. 3. Microtubules in *nim*T23, *mip*A$^+$ and *nim*T23, *mip*Ad1 germlings blocked in G_2. (a and b) The same field containing two *nim*T23, *mip*A$^+$ (SO26) germlings that have been germinated at a restrictive temperature and are blocked in G_2. (a) A phase contrast micrograph and (b) an immunofluorescence micrograph showing microtubules as revealed by an anti-β-tubulin antibody. A normal array of cytoplasmic microtubules is present. (c and d) A *nim*T23, *mip*Ad1 germling also germinated at a restrictive temperature and blocked in G_2. An array of cytoplasmic microtubules indistinguishable from those in strains with functional γ-tubulin is present as shown by anti-β-tubulin staining in d. Bar, 10 μm.

ments each with *nim*A5 and *nim*T23 strains, sample size 350 germlings for each experiment). The number of nuclei in these germlings doubled again by two hours after the shift to the permissive temperature. In the ts$^-$, *mip*Ad1 germlings, however, there was a complete blockage of nuclear division (no nuclear divisions in a total of 10,500 germlings examined at 20 minutes and later time points) over the time course of the experiments. Nuclear divisions, often unequal, were very rarely observed after prolonged incubations.

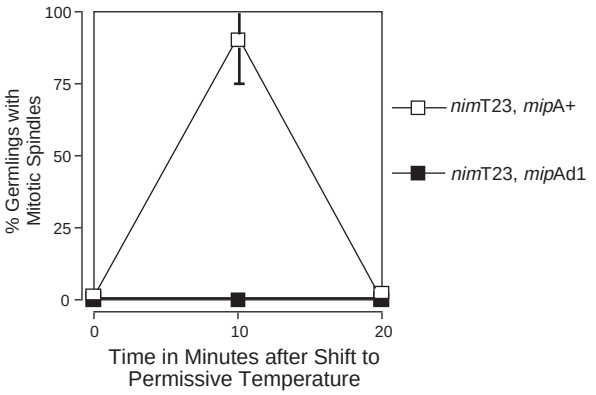

Fig. 4. Spindle mitotic indices in block/release experiments. Conidia of SO26 (*nim*T23, *mip*A$^+$) and H9 (*nim*T23, *mip*Ad1) were germinated at a restrictive temperature and shifted to a permissive temperature at time 0. Samples were taken immediately before the shift (0 time point) and at intervals after the shift. Values show the percentage of germlings with mitotic spindles as judged by immunofluorescence microscopy. Each value is an average of three experiments with 350 germlings scored for each strain at each time point in each experiment. Error bars denote 95% confidence limits and in instances in which error bars are not shown 95% confidence limits fell within the boxes used to denote the values.

We next asked if mitotic spindles were present in mitotic nuclei of *mip*Ad1 germlings. In wild-type germlings, interphase cytoplasmic microtubules disassemble at the onset of mitosis and mitotic spindles assemble. As the mitotic spindle elongates, cytoplasmic microtubules begin to reassemble and form a normal cytoplasmic array at the end of mitosis. As expected, at the G_2 block points the ts$^-$, *mip*A$^+$ germlings contained an array of cytoplasmic microtubules (Fig. 3a,b). At 10 minutes after the shift to the permissive temperature, cytoplasmic microtubules had disassembled and mitotic spindles (see Figs 4, 5a-c) had formed in the vast majority of germlings. At 20 minutes, mitosis had been completed in the great majority of germlings. Cytoplasmic microtubules had reassembled and spindles had disassembled.

Perhaps surprisingly, we found that cytoplasmic microtubules were present in ts$^-$, *mip*Ad1 germlings at the restrictive temperature (Fig. 3c,d). At 10 minutes after the shift, cytoplasmic microtubules had disassembled but mitotic spindles were completely absent (Figs 4, 5d-f) (no spindles in 2,100 germlings examined at this time point). As a control for antibody penetration, we used a monoclonal antibody to histone H1. The antibody stained condensed chromosomes in *nim*T23, *mip*A$^+$ control germlings (Fig. 5c) and in *nim*T23, *mip*Ad1 germlings (Fig. 5f) even though spindles were absent from the latter. There was thus no problem with antibody penetration.

The ts$^-$, *mip*Ad1 germlings still had no spindles nor cytoplasmic microtubules at 20 minutes after the shift to the permissive temperature. Most ts$^-$, *mip*Ad1 germlings did regain one or two cytoplasmic microtubules between 30 and 60 minutes after release and over 80% of the germlings had some cytoplasmic microtubules by 60 minutes. These cytoplasmic microtubules were quite different from those seen in normal germlings, however. There were usually only one or two of them and they were often curled or exhibited other morpho-

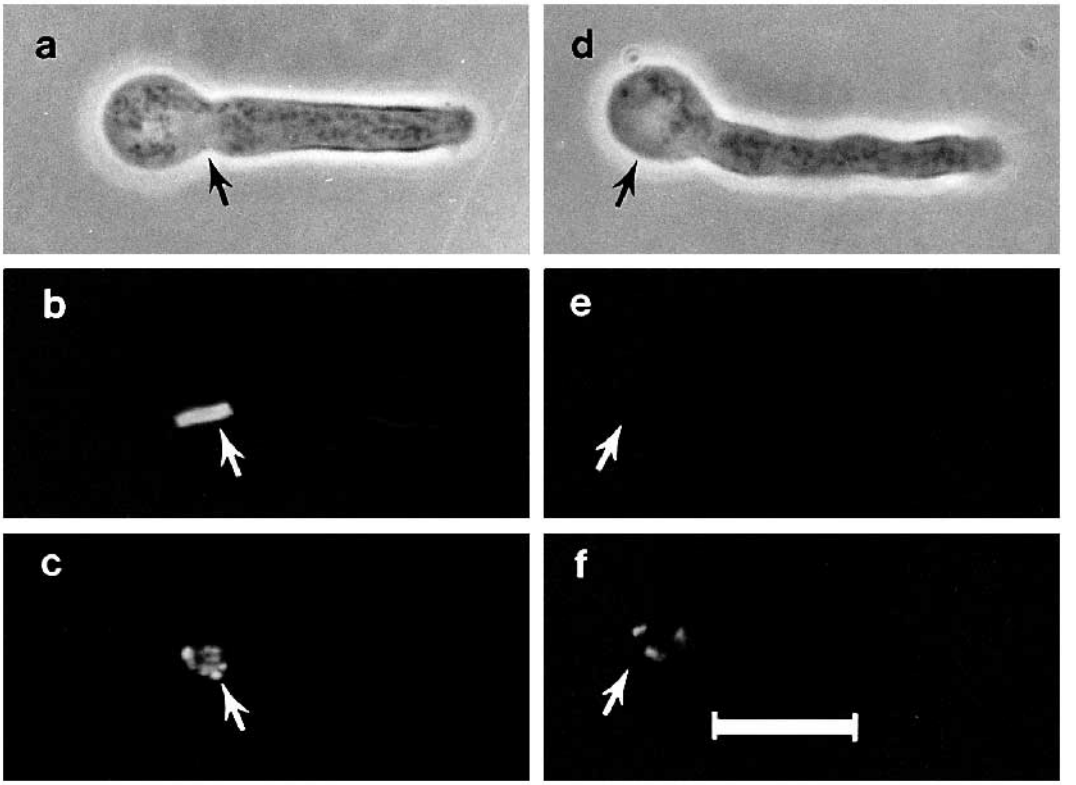

Fig. 5. Failure of mitotic spindle formation in *mip*Ad1 germlings. (a-c) A *nim*T23, *mip*A$^+$ germling that has been blocked in G_2 and the block released by shifting to a permissive temperature for 10 minutes. Anti-α-tubulin staining (b) shows that the cytoplasmic microtubule array has disassembled and a mitotic spindle (arrow) has formed. Chromatin has condensed as shown by anti-histone H1 staining (c). (d-f) A *nim*T23, *mip*Ad1 germling that has also been blocked in G_2 then shifted to the permissive temperature for 10 minutes. Staining with an anti-α-tubulin antibody (e) reveals a complete absence of microtubules. This indicates that cytoplasmic microtubules have disassembled but no mitotic spindle has formed. Chromatin has condensed as shown by anti-histone H1 staining (f). The chromosomes in this germling were spread through a greater volume than those in c, perhaps because they were not attached to a mitotic spindle. As a result some chromosomes are not in the plane of focus. Bar, 10 μm.

logical abnormalities. They were similar to the abnormal arrays of microtubules seen previously in unsynchronized γ-tubulin disruptant germlings (See Fig. 5F in Oakley et al., 1990). By three hours after release, most ts$^-$, *mip*Ad1 germlings contained interphase chromatin and a few cytoplasmic microtubules. Spindles were completely absent at all stages.

Effects of a γ-tubulin disruption on spindle pole body replication, separation and MPM-2 reactivity

We found that, as expected, MPM-2 and γ-tubulin antibodies both stained the SPBs in control *mip*A$^+$ germlings (Figs 6b, 7e). As previously reported (Engle et al., 1988), MPM-2 staining was present only in G_2 and M germlings. γ-Tubulin staining was present throughout the cell cycle. γ-Tubulin staining was absent from all SPBs in γ-tubulin disruptant (*mip*Ad1) germlings but was present in parental *mip*A$^+$ swollen conidia in the same preparation (Fig. 6f). These results confirm that γ-tubulin is eliminated or reduced to a level undetectable by immunofluorescence microscopy in γ-tubulin disruptant germlings. In spite of the absence of γ-tubulin, however, SPBs were MPM-2 reactive (Fig. 6e).

The fact that MPM-2 reactivity was maintained in the absence of γ-tubulin allowed us to examine SPB behavior in γ-tubulin disruptants. In control ts$^-$, *mip*A$^+$ germlings blocked in G_2 by germination at the restrictive temperature, SPBs were MPM-2 reactive. The SPBs had duplicated (G_1 nuclei have a single SPB; B. R. Oakley and N. R. Morris, unpublished) and the two SPBs were always immediately adjacent. Upon shifting to permissive temperature the SPBs remained MPM-2 reactive as nuclei entered mitosis and the two SPBs were at opposite ends of the mitotic spindle (Fig. 7c-e). By 20 minutes after the shift to the permissive temperature, nuclei had entered interphase and SPBs were no longer MPM-2 reactive.

In ts$^-$, *mip*Ad1 germlings blocked in G_2, SPBs were also MPM-2 reactive and were present as pairs (Fig. 7a,b). This indicates that SPB replication had occurred in the disruptants. Upon shifting to the permissive temperature, SPBs remained MPM-2 reactive but, as no spindles formed, the SPB pairs remained close together (Fig. 7f-h). The SPBs remained MPM-2 reactive in the γ-tubulin disruptants for the duration of the mitotic block (i.e. germlings with condensed chromatin had MPM-2 reactive SPBs). By 60 minutes after the shift to the permissive temperature, the SPBs in some nuclei had separated from each other. Approximately 50% of the ts$^-$ *mip*Ad1 germlings had one or both SPBs associated with cytoplasmic microtubules by this time point, but this number varied greatly among experiments. By three hours after the shift, the nuclei in the ts$^-$, *mip*Ad1 germlings had entered interphase without dividing and SPBs were no longer MPM-2 reactive.

In *mip*Ad1 germlings from H1, H7 and H9 germinated on selective medium at a permissive temperature (and therefore asynchronous), we found that SPBs were MPM-2 reactive in approximately 30% of the germlings. This is similar to the percentage of wild-type germlings with MPM-2 reactivity when they are grown asynchronously (Engle et al., 1988). There were obviously multiple SPBs in a few of the *mip*Ad1 germlings, probably reflecting multiple rounds of SPB replication in the absence of nuclear division. Some nuclei had as many as eight separate MPM-2 reactive spots. The great majority of *mip*Ad1 germlings had only one or two MPM-2 reactive spots, but in

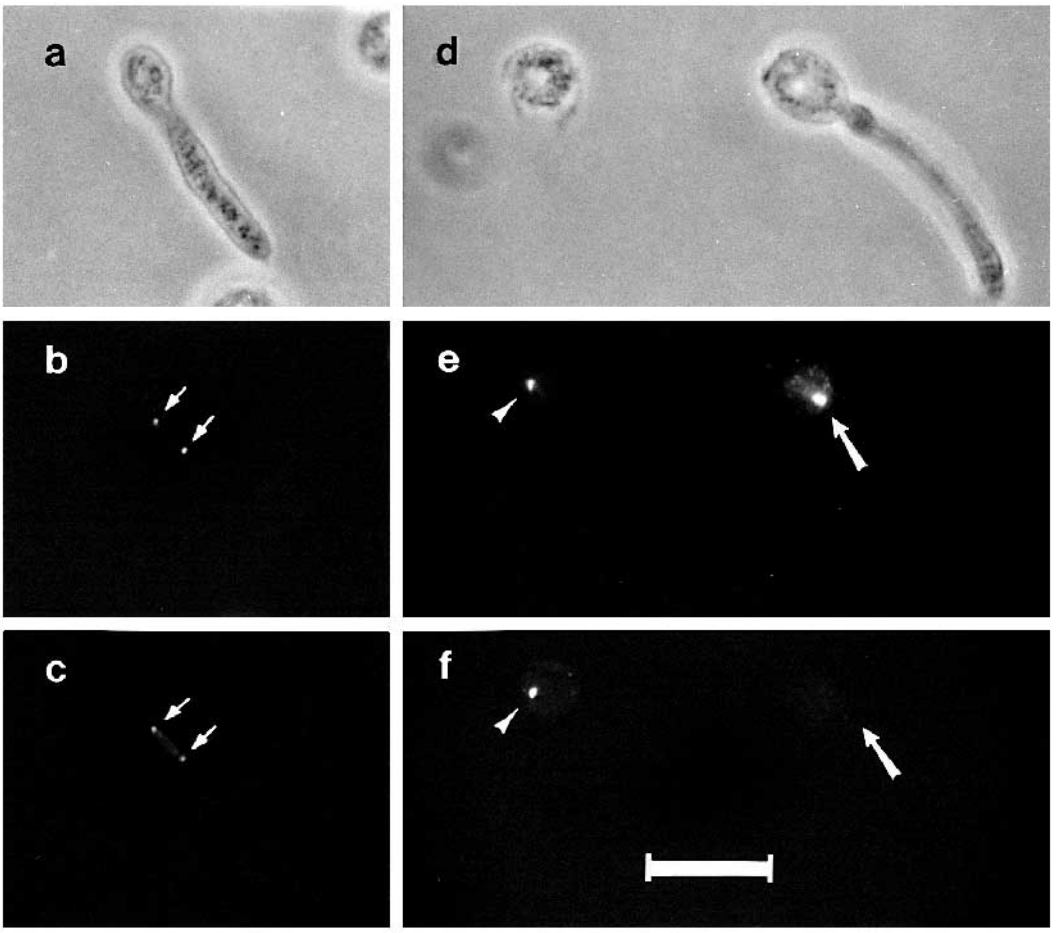

Fig. 6. Spindle pole bodies of *mip*Ad1 germlings lack detectable γ-tubulin but retain MPM-2 reactivity. (a-c) A control *nim*A5, *mip*A$^+$ strain (SO6) grown in non-selective medium. We have chosen a mitotic germling to illustrate SPB staining. (a) A phase contrast micrograph. (b) Staining with MPM-2 antibody. Though it is not shown, a mitotic spindle is present and each of the two spindle pole bodies (one at each end of the mitotic apparatus) is MPM-2 reactive. The spindle pole bodies also stain with an anti-γ-tubulin antibody (c). (d-f) Conidia from heterokaryon H9 germinated in selective medium. At the left is a uninucleate *nim*A5, *mip*A$^+$ conidium. It has swollen but not germinated. Its spindle pole body is MPM-2 reactive (arrowhead in e) and stains with an anti-γ-tubulin antibody (arrowhead in f). At the right is a *nim*A5, *mip*Ad1 germling. Its spindle pole body is MPM-2 reactive (arrow in e) but shows no reactivity with the anti-γ-tubulin antibody (arrow in f). The nucleoplasm is also somewhat MPM-2 reactive. The brightness of the MPM-2 staining of the SPB in the *nim*A5, *mip*A$^+$ conidium and in the *nim*A5, *mip*Ad1 germling is due in part to the fact that the SPBs have replicated in each case (though the two daughter SPBs are not resolved from each other in this micrograph). Bar, 10 μm.

many cases these were stained brightly and may have been multiple, unseparated SPBs (data not shown).

DISCUSSION

We have developed a modification of the heterokaryon rescue technique that has allowed us to examine the effects of a γ-tubulin disruption in the first cell cycle after germination. This has permitted us to distinguish between primary phenotypes of the disruption and secondary phenotypes caused by progression through multiple cell cycles in the absence of functional γ-tubulin. Our results demonstrate clearly that γ-tubulin is essential for the assembly of mitotic spindle microtubules in *A. nidulans*. The completeness of the blockage of mitotic spindle formation was extraordinary. We examined more than 10,000 γ-tubulin disruptant germlings that were in mitosis (i.e. with condensed chromosomes) without finding a single spindle. We observed mitotic spindles in almost 2,000 germlings of control strains in the same experiments so the absence of mitotic spindles was clearly not due to any inability to preserve and stain spindles. The fact that virtually identical results were obtained using either *nim*A5 or *nim*T23 to synchronize the cell cycle appears to rule out the possibility that the blockage of spindle formation was due to specific interactions between *mip*Ad1 and *nim*A5 or *nim*T23. The presence of cytoplasmic microtubules at the G_2 block point demonstrates that assembly-competent tubulin was present and that the inhibition of spindle formation was not due to any possible effects of the γ-tubulin disruption on the levels of assembly-competent α- and β-tubulin. The completeness of the blockage of spindle microtubule assembly supports the hypothesis that γ-tubulin nucleates the assembly of spindle microtubules.

How can our results be reconciled with the data from *S. cerevisiae* and *D. melanogaster* that suggest that spindle microtubules can assemble in the absence of functional γ-tubulin (Sunkel et al., 1995; Sobel and Snyder, 1995; Spang et al., 1996; Marschall et al., 1996)? With respect to *S. cerevisiae*, one possibility raised by Spang et al. (1996) is that the ts$^-$ *TUB4* alleles may disrupt some but not all of the functions of Tub4p. For example, a *TUB4* allele might disrupt the attachment of Tub4p to the SPB without disrupting its ability to nucleate microtubule assembly. It is also important to remember that the *S. cerevisiae TUB4* product is remarkably divergent from other γ-tubulins. Indeed, it shares less identity with other γ-tubulins than α-tubulins share with β-tubulins and the designation ε-tubulin was originally proposed for this protein (Burns, 1995). A *TUB4* mutation was not comple-

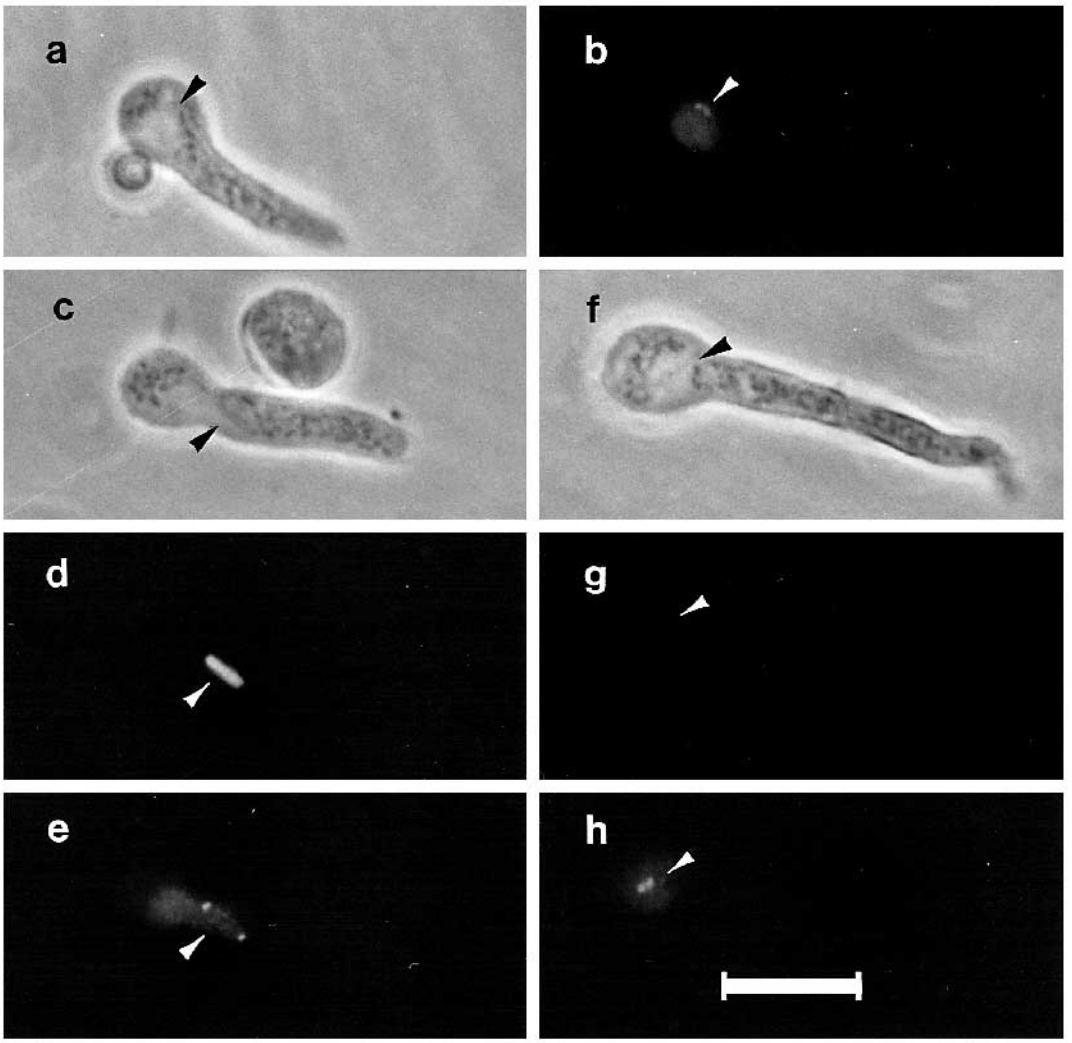

Fig. 7. Spindle pole bodies and mitotic spindles in a block/release experiment. (a and b) A *nim*A5, *mip*Ad1 germling blocked in G_2 by germination at a restrictive temperature. (b) MPM-2 staining. The replicated SPB is visible (arrowhead) as two adjacent MPM-2 reactive spots. The nucleoplasm is slightly MPM-2 reactive. (c-e) A *nim*A5, *mip*A^+ germling that has been blocked in G_2 by germination at a restrictive temperature then shifted to a permissive temperature for eight minutes to release the block. The germling has entered mitosis. A spindle is present (arrowhead in d) as shown with anti-α-tubulin staining and the SPBs are at the ends of the mitotic spindle as shown by MPM-2 staining (e). (f-h) A *nim*A5, *mip*Ad1 germling that has also been blocked in G_2 before shifting to the permissive temperature for eight minutes. Anti-α-tubulin staining reveals that no mitotic spindle is present (g) and MPM-2 staining reveals that the replicated SPBs remain close together (h). Bar, 10 μm.

mented by human or *Xenopus* γ-tubulins (Spang et al., 1996; Marschall et al., 1996) nor was an *S. pombe* γ-tubulin null mutation complemented by *TUB4* (Marschall et al., 1996). More typical γ-tubulins are functionally conserved among phylogenetically diverse organisms. A human γ-tubulin functions in *S. pombe*, for example, complementing a disruption of the *S. pombe* γ-tubulin gene (Horio and Oakley, 1994). We favor the view put forth by Spang et al. (1996) that the *TUB4* product is a highly divergent γ-tubulin adapted to the specialized microtubule attachment structures of *S. cerevisiae*. Because of its great divergence from the γ-tubulins of other organisms, we feel that it is unlikely to share all of the functions of other γ-tubulins. While Tub4p may not nucleate the assembly of mitotic spindle microtubules, we believe that it is likely that more typical γ-tubulins do play such a role.

With respect to the *D. melanogaster* data, Sunkel et al. (1995) studied the phenotype of a P-element insertion into the 5′ untranslated region of the *γ-TUB23C* locus and found that the nucleation of spindle microtubules was reduced but not eliminated. They used western blots to demonstrate that the level of γ-tubulin was reduced below the level of detection in larval brain cells. The possibility was not ruled out, however, that the P-element insertion results in a severe hypomorph rather than a null mutation. This could leave a small amount of functional γ-tubulin, not detected on western blots, which could nucleate the assembly of some spindle microtubules. It is also important to note that a second γ-tubulin gene has been identified in *D. melanogaster* (Zheng et al., 1991). The product of this gene has now been designated γTub37CD. Sunkel et al. did not evaluate the reactivity of their antibodies with γTub37CD. This leaves open the possibility that some γTub37CD was present in the cells studied by Sunkel et al. and may have been responsible for the observed assembly of mitotic spindle microtubules. Finally, chromatin promotes microtubule assembly in meiotic *D. melanogaster* cells (see discussion and references in Matthies et al., 1996) and it is conceivable that it may also promote some assembly in mitotic cells.

Although we found that the assembly of spindle microtubules was abolished completely in *A. nidulans* γ-tubulin disruptants, the situation with respect to cytoplasmic microtubules was more complex. An apparently normal array of cytoplasmic microtubules was present at the G_2 block point. When the block was released, the cytoplasmic microtubules disassembled as in control germlings, but when germlings entered interphase (after a block in mitosis), normal microtubule arrays did not reassemble. Instead, microtubules were much fewer in number than in controls and were curled or exhibited other

morphological abnormalities. Normal arrays of cytoplasmic microtubules were not noted in a previous study of unsynchronized γ-tubulin disruptant germlings (Oakley et al., 1990). This was undoubtedly because, as discussed above, it was not possible in unsynchronized material to distinguish γ-tubulin disruptant germlings until they had passed through at least one cell cycle (at which time normal cytoplasmic arrays had presumably disassembled and not reformed). The abnormal microtubule arrays seen in this study are similar to those seen previously (Oakley et al., 1990), but they were seen in fewer germlings in the previous study.

What do these results tell us about the role of γ-tubulin in nucleating the assembly of cytoplasmic microtubules? At face value, they appear to show that γ-tubulin is not required for nucleation of cytoplasmic microtubules. This possibility is consistent with the finding that cytoplasmic microtubules are present in *D. melanogaster* cells depleted of γ-tubulin and in *S. cerevisiae TUB4* mutants under restrictive conditions. The caveats that apply to the results in *D. melanogaster* and *S. cerevisiae* for spindle formation also apply to cytoplasmic microtubule assembly, however, and it is probably premature to conclude that γ-tubulin does not play a role in the assembly of cytoplasmic microtubules.

With respect to our results, there are alternate explanations as well. It is conceivable, with respect to the normal microtubule array in G_2-blocked cells, that pre-formed microtubules may be present in conidia even before germination. Microtubules are seen in wild-type and *mip*Ad1 germinating conidia as soon as the conidial walls can be made permeable to antibodies. These microtubules could have formed in the conidiophore of the parental heterokaryon and thus γ-tubulin in the conidium would not be required to nucleate their assembly. This might explain why normal microtubule arrays are found in the first cell cycle but are never seen again after they disassemble as germlings enter mitosis. We also cannot rule out the possibility that a very small amount of γ-tubulin was carried over into the γ-tubulin disruptant conidia from the parental heterokaryon. A few molecules of γ-tubulin (presumably on the cytoplasmic face of the SPBs) might conceivably be sufficient to nucleate the assembly of the observed cytoplasmic microtubules. If any γ-tubulin was carried over, however, it was not detectable by immunofluorescence and it was insufficient to nucleate any detectable spindle microtubule assembly. We were unable to use western blotting to confirm the absence of γ-tubulin from disruptant germlings because, as mentioned, the γ-tubulin disruptant heterokaryons necessarily produce a mixture of γ-tubulin disruptant and parental conidia. While γ-tubulin disruptant germlings are easily distinguished in the microscope from the swollen but ungerminated parental conidia, it is not possible to separate them physically. Any protein preparation from γ-tubulin disruptant germlings thus contains normal γ-tubulin from the parental conidia present.

What accounts for the assembly of a reduced number of microtubules in the γ-tubulin disruptants as they re-enter interphase? If γ-tubulin played no role in cytoplasmic microtubule assembly, one would expect a normal array of cytoplasmic microtubules to assemble at this point (barring unknown consequences of cell cycle perturbation). We observe some microtubule assembly but far less than normal. One possibility is that the assembly of these microtubules is nucleated by a very small amount of carryover γ-tubulin, diminished by turnover in the period after the release of the G_2 block. A second possibility is that we are observing non-nucleated assembly of microtubules. MTOCs are not necessary for microtubule assembly in vitro and some spontaneous assembly might occur in vivo in the absence of functional nucleation sites on the SPB.

Our data provide useful information on the role of γ-tubulin in the cell cycle. The fact that the ts⁻ *mip*Ad1 strains entered mitosis when shifted from restrictive to permissive temperatures indicates that functional γ-tubulin is not required for the G_2 to M transition. Since the mitotic index was very high immediately after the shift, virtually all of these germlings must have been blocked in G_2 at the restrictive temperature. Conidia are arrested in G_0 before germination and thus enter the cell cycle in G_1 (Bergen and Morris, 1983). Since the ts⁻ *mip*Ad1 germlings arrest in G_2 at the restrictive temperature, functional γ-tubulin is apparently not required for the progression from G_1 through S to G_2.

The transition out of mitosis was greatly delayed in γ-tubulin disruptants. This prolonged blockage in mitosis was, in a sense, expected because most reagents and mutations that inhibit spindle formation cause a blockage in mitosis and thus an elevated CMI. It is a significant finding, however, because previous γ-tubulin disruption and depletion experiments have not revealed a substantially elevated CMI (Oakley et al., 1990; Horio et al., 1991, Sunkel et al., 1995). Interestingly, chromosomes do eventually decondense, but nuclear division does not occur. Chromosomal decondensation is apparently quite gradual in the γ-tubulin disruptants because we see many nuclei with partially decondensed chromatin 90-120 minutes after the shift to permissive temperature. In wild-type cells the entire process of mitosis lasts approximately five minutes and the process of chromosomal decondensation cannot last more than 1-2 minutes. These results suggest that in the absence of γ-tubulin, the checkpoint that controls the M to interphase transition is eventually overcome without nuclear division, but the transition is much slower than normal.

The data we have reported here and the previous data of Oakley et al. (1990) present a paradox. In non-temperature-sensitive *mip*Ad1 germlings and in ts⁻, *mip*Ad1 germlings grown at permissive temperatures, CMIs were similar to the wild-type (about 5%). This suggests that there is no prolonged mitotic block in asynchronous γ-tubulin disruptants. Our block-release experiments, however, revealed a block in mitosis of 60-120 minutes in strains carrying *mip*Ad1. In a strain carrying *nim*T23, the entire cell cycle requires 95 minutes at a permissive temperature of 32°C, a time very similar to the cell cycle time for a wild-type strain at the same temperature (Bergen and Morris, 1983). Of the 95 minute cell cycle, approximately 90 minutes is spent in interphase. If, in unsynchronized germlings carrying *mip*Ad1, the cell cycle proceeded normally at this temperature except for a 90 minute blockage in mitosis, one would expect a chromosome mitotic index of approximately 50% as interphase and mitosis would each take about 90 minutes. Instead, we observe a CMI of only about 5%. This paradox is probably not unique to *A. nidulans* because in *D. melanogaster* cells depleted of γ-tubulin actually have a lower mitotic index than comparable cells in a control strain (Sunkel et al., 1995).

At this point we can only speculate as to the cause of the lower than expected CMI. The most obvious difference between the synchronized and unsynchronized germlings is

that in the synchronized material we were able to observe events of the first cell cycle after germination, whereas in the unsynchronized material it is likely that most of the germlings had gone through more than one cell cycle. Perhaps in unsynchronized material the second and subsequent cell cycles were abnormal in some ways. After all, nuclear division was blocked in these germlings and the nuclei entered interphase without dividing. These events might perturb subsequent cell cycles, reducing the length of the mitotic block and/or lengthening interphase and resulting in a relatively low CMI.

The presence of MPM-2 reactive antigens at SPBs in γ-tubulin disruptants in which γ-tubulin is not detectable, demonstrates that, if γ-tubulin is MPM-2 reactive at all, it accounts for, at most, a small fraction of the MPM-2 reactivity of normal SPBs. These data also demonstrate that the MPM-2 reactive material does not require γ-tubulin for localization at the SPB, i.e. the MPM-2 reactive material must be bound to the SPB by something other than γ-tubulin. These data provide an important in vivo correlation for the in vitro results of Félix et al. (1994) who found that under certain conditions sperm centrioles can acquire MPM-2 reactivity in *Xenopus* extracts without acquiring γ-tubulin. Additionally, Masuda et al. (1992) have demonstrated that MPM-2 reactivity of *S. pombe* SPBs is not sufficient for microtubule nucleation in vitro and Félix et al. (1994) have obtained similar results in vitro for centrosomes. Our data demonstrate clearly that MPM-2 reactivity in the absence of functional γ-tubulin is insufficient for assembly of the mitotic spindle from SPBs in *A. nidulans* in vivo. Moreover, in our system all of the necessary mitotic kinases (p34cdc2, NIMA, and any other, as yet unknown, kinases) must be present and functional because spindles form readily under the same conditions in strains that contain functional γ-tubulin.

A useful consequence of the MPM-2 reactivity in γ-tubulin disruptants is that it has allowed us to determine that SPB replication occurs in the absence of functional γ-tubulin but SPB separation is inhibited. Because of the small size of SPBs it was not possible to determine, by light microscopy, if *A. nidulans* SPBs that assemble in the absence of functional γ-tubulin are morphologically normal. SPB replication also occurs in *TUB4* mutants of *S. cerevisiae* (Spang et al., 1996; Marschall et al., 1996), but SPB separation is much less inhibited than in *A. nidulans*.

We thank Dr M. Katherine Jung for advice on immunofluorescence and for critically reading the manuscript, Elizabeth Oakley for helpful discussions and critical reading of the manuscript, Gary Grumbling for assistance with statistical and graphing software, Dr Fred Sack and the Ohio State University Department of Plant Biology for use of their dark room, and Dr Peter Mirabito for discussions on MPM-2 staining. Supported by NIH grants GM31837 (to B.R.O.) and GM42564 (to S.A.O.).

REFERENCES

Bergen, L. G. and Morris, N. R. (1983). Kinetics of the nuclear division cycle of *Aspergillus nidulans*. *J. Bacteriol.* **156**, 155-160.

Bergen, L. G., Upshall, A. and Morris, N. R. (1984). S-phase, G2, and nuclear division mutants of *Aspergillus nidulans*. *J. Bacteriol.* **159**, 114-119.

Brinkley, B. R. (1985). Microtubule organizing centers. *Annu. Rev. Cell Biol.* **1**, 145-172.

Burns, R. G. (1995). Identification of two new members of the tubulin family. *Cell Motil. Cytoskel.* **31**, 255-258.

Centonze, V. E. and Borisy, G. G. (1990). Nucleation of microtubules from mitotic centrosomes is modulated by a phosphorylated epitope. *J. Cell Sci.* **95**, 405-411.

Davis, F. M., Tsao, T. H., Fowler, S. K. and Rao, P. N. (1983). Monoclonal antibodies to mitotic cells. *Proc. Nat. Acad. Sci. USA* **80**, 2926-2930.

Engle, D. B., Doonan, J. H. and Morris, N. R. (1988). Cell-cycle modulation of MPM-2-specific spindle pole body phosphorylation in *Aspergillus nidulans*. *Cell Motil. Cytoskel.* **10**, 432-437.

Félix, M.-A., Antony, C., Wright, M. and Maro, B. (1994). Centrosome assembly in vitro: Role of γ-tubulin recruitment in Xenopus sperm aster formation. *J. Cell Biol.* **124**, 19-31.

Horio, T., Uzawa, S., Jung, M. K., Oakley, B. R., Tanaka, K. and Yanagida, M. (1991). The fission yeast γ-tubulin is essential for mitosis and is localized at two different microtubule organizing centers. *J. Cell Sci.* **99**, 693-700.

Horio, T. and Oakley, B. R. (1994). Human γ-tubulin functions in fission yeast. *J. Cell Biol.* **126**, 1465-1473.

Joshi, H. C., Palacios, M. J., McNamara, L. and Cleveland, D. W. (1992). γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature* **356**, 80-83.

Kalt, A. and Schliwa, M. (1993). Molecular components of the centrosome. *Trends Cell Biol.* **3**, 118-127.

Kimble, M. and Kuriyama, R. (1992). Functional components of microtubule-organizing centers. *Int. Rev. Cytol.* **136**, 1-50.

Li, Q. and Joshi, H. C. (1995). γ-Tubulin is a minus end-specific microtubule binding protein. *J. Cell Biol.* **131**, 207-214.

Marschall, L. G., Jeng, R. L., Mulholland, J. and Stearns, T. (1996). Analysis of Tub4p, a yeast γ-tubulin-like protein: Implications for microtubule-organizing center function. *J. Cell Biol.* **134**, 443-454.

Masuda, H., Sevik, M. and Cande, W. Z. (1992). In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast *Schizosaccharomyces pombe*: Cell cycle-dependent activation in Xenopus cell-free extracts. *J. Cell Biol.* **117**, 1055-1066.

Masuda, H. and Shibata, T. (1996). Role of γ-tubulin in mitosis-specific microtubule nucleation from the *Schizosaccharomyces pombe* spindle pole body. *J.Cell Sci.* **109**, 165-177.

Matthies, H. J. G., McDonald, H. B., Goldstein, L. S. B. and Theurkauf, W. E. (1996). Anastral meiotic spindle morphogenesis: Role of the Non-Claret Disjunctional kinesin-like protein. *J. Cell Biol.* **134**, 455-464.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B. and Agard, D. A. (1995). Microtubule nucleation by γ-tubulin containing rings in the centrosome. *Nature* **378**, 638-640.

Oakley, B. R. and Morris, N. R. (1980). Nuclear movement is β-tubulin-dependent in Aspergillus nidulans. *Cell* **19**,255-262.

Oakley, B. R. and Morris, N. R. (1983). A mutation in *Aspergillus nidulans* that blocks the transition from interphase to prophase. *J. Cell Biol.* **96**, 1155-1158.

Oakley, B. R. and Rinehart, J. E. (1985). Mitochondria and nuclei move by different mechanisms in *Aspergillus nidulans*. *J. Cell Biol.* **101**, 2392-2397.

Oakley, B. R., Oakley, C. E., Yoon, Y. and Jung, M. K. (1990). γ Tubulin is a component of the spindle-pole-body that is essential for microtubule function in Aspergillus nidulans. *Cell* **61**, 1289-1301.

Oakley, B. R. (1992). γ-Tubulin: the microtubule organizer? *Trends Cell Biol.* **2**, 1-5.

O'Connell, M. J., Osmani, A. H., Morris, N. R. and Osmani, S. A. (1992). An extra copy of *nim*E^{cyclinB} elevates pre-MPF levels and partially suppresses mutation of *nim*T^{cdc25} in *Aspergillus nidulans*. *EMBO J.* **11**, 2139-2140.

Osmani, S. A., May, G. S. and Morris, N. R. (1987). Regulation of the mRNA levels of *nimA*, a gene required for the G2-M transition in *Aspergillus nidulans*. *J. Cell Biol.* **104**, 1495-1504.

Osmani, S. A., Engle, D. B., Doonan, J. H. and Morris, N. R. (1988a). Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell* **52**, 241-251.

Osmani, S. A., Pu, R. T. and Morris, N. R. (1988b). Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell* **53**, 237-244.

Osmani, A. H., O'Donnell, K., Pu, R. T. and Osmani, S. A. (1991). Activation of the *nim*A protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the *bim*E checkpoint. *EMBO J.* **10**, 2669-2679.

Rose, M. D., Biggins, S. and Satterwhite, L. L. (1993). Unravelling the tangled web at the microtubule-organizing center. *Curr. Opin. Cell Biol.* **5**, 105-115.

Sobel, S. G. and Snyder, M. (1995). A highly divergent gamma-tubulin gene is

essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 1775-1788.

Spang, A., Geissler, S., Grein, K. and Schiebel, E. (1996). Gamma-tubulin-like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle formation. *J. Cell Biol.* **134**, 429-441.

Stearns, T., Evans, L. and Kirschner, M. (1991). γ Tubulin is a highly conserved component of the centrosome. *Cell* **65**, 825-836.

Stearns, T. and Kirschner, M. (1994). In vitro reconstitution of centrosome assembly and function: The central role of γ-tubulin. *Cell* **76**, 623-637.

Sunkel, C. E, Gomes, R., Sampaio, P., Perdigao, J. and Gonzalea, C. (1995). γ-Tubulin is required for the structure and function of the microtubule organizing centre in Drosophila neuroblasts. *EMBO J.* **14**, 28-36.

Vandré, D. D., Davis, F. M., Rao, P. N. and Borisy, G. G. (1984). Phosphoproteins are components of mitotic microtubule organizing centers. *Proc. Nat. Acad. Sci. USA* **81**, 4439-4443.

Verde, F., Labbe, J. C., Doree, M. and Karsenti, E. (1990). Regulation of microtubule dynamics by Cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature* **343**, 233-238.

Zheng, Y., Oakley, C. E. and Oakley, B. R. (1991). Identification of a second gamma-tubulin gene in *Drosophila melanogaster*. *J. Cell Biol.* **115**, 382a.

Zheng, Y., Wong, M. L., Alberts, B. and Mitchison, T. (1995). Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378**, 578-583.

(Received 11 July 1996 - Accepted 18 December 1996)