Two Related Kinesins, *klp5*⁺ and *klp6*⁺, Foster Microtubule Disassembly and Are Required for Meiosis in Fission Yeast

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Submitted June 20, 2001; Revised August 15, 2001; Accepted September 19, 2001
Monitoring Editor: Lawrence S. Goldstein

The kinesin superfamily of microtubule motor proteins is important in many cellular processes, including mitosis and meiosis, vesicle transport, and the establishment and maintenance of cell polarity. We have characterized two related kinesins in fission yeast, *klp5*⁺ and *klp6*⁺, that are amino-terminal motors of the KIP3 subfamily. Analysis of null mutants demonstrates that neither *klp5*⁺ nor *klp6*⁺, individually or together, is essential for vegetative growth, although these mutants have altered microtubule behavior. *klp5*Δ and *klp6*Δ are resistant to high concentrations of the microtubule poison thiabendazole and have abnormally long cytoplasmic microtubules that can curl around the ends of the cell. This phenotype is greatly enhanced in the cell cycle mutant *cdc25*–22, leading to a bent, asymmetric cell morphology as cells elongate during cell cycle arrest. Klp5p-GFP and Klp6p-GFP both localize to cytoplasmic microtubules throughout the cell cycle and to spindles in mitosis, but their localizations are not interdependent. During the meiotic phase of the life cycle, both of these kinesins are essential. Spore viability is low in homozygous crosses of either null mutant. Heterozygous crosses of *klp5*Δ with *klp6*Δ have an intermediate viability, suggesting cooperation between these proteins in meiosis.

INTRODUCTION

The microtubule cytoskeleton is required for a variety of cellular functions in eukaryotes, including the organization of cellular organelles, the establishment and maintenance of cell polarity, and chromosome movement in mitosis and meiosis. Microtubule dynamics and organization are controlled in part by proteins that associate with polymerized tubulin: microtubule-associated proteins and the motor proteins kinesin and dynein (reviewed in Hunter and Wordeman, 2000). The kinesin motor proteins constitute a superfamily of related molecules defined by a conserved motor domain of ~320 amino acids that displays ATPase activity and microtubule binding; together these produce motility. Kinesins are classified into subfamilies based on motor domain sequence similarities, whereas the sequences outside the motor domain often lack significant similarity, even within a subfamily (reviewed in Goldstein and Philp, 1999).

The fission yeast, *S. pombe*, is an attractive system for the study of kinesin function, given its relatively small complement of motors together with its suitability for detailed experimentation, e.g., one can efficiently manipulate its genes through molecular genetics. The study of motor enzymes in fission yeast is further facilitated by the elaborate and dynamic microtubule cytoskeleton found in these cells, which is readily visible by light microscopy (reviewed by Hagan, 1998). Interphase cells have an extensive array of cytoplasmic microtubules organized as 3 to 8 bundles that run essentially parallel to the long axis of the cell. These microtubules appear to be organized with their plus ends at the cell tips, while the minus ends congregate in a small region of interdigitation around the nucleus, which lies at the center of the cell (Drummond and Cross, 2000; Tran *et al.*, 2001; reviewed by Hagan, 1998). Moreover, these microtubules undergo dynamic instability with parameters similar to those described for metazoan systems (Drummond and Cross, 2000; Tran *et al.*, 2001). Unlike in budding yeast, these microtubules are essential for organizing vesicular organelles (Ayscough *et al.*, 1993; Yaffe *et al.*, 1996; Hagan and Yanagida, 1997) and maintaining cell polarity (reviewed in Mata and Nurse, 1998; Chang, 2001). The most dramatic change in microtubule organization occurs as the cells enter

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Abbreviations used: DAPI, 4', 6-diamino-2-phenylindole; KLP, kinesin-like protein; GFP, green fluorescent protein; PCR, polymerase chain reaction; RT, reverse transcriptase; TBZ, thiabendazole; YES, yeast extract with supplements.

Table 1. Strains used in this study

Strain	Genotype	Source
42	<i>ade6-M210, leu1-32, ura4-D18, h-</i>	Nurse 557
45	<i>ade6-M216, leu1-32, ura4-D18, h+</i>	Nurse 556
51	<i>cdc2-33, ade6-M210, leu1-32, ura4-D18, h+</i>	This study
61	<i>wee1-50, leu1-32, ura4-D18, h+</i>	This study
99	<i>ade6-M210, his3-D1, leu1-32, ura4-D18, h-</i>	Browning
100	<i>ade6-M216, his3-D1, leu1-32, ura4-D18, h+</i>	Browning
105	<i>ade6-M210, his2-1, leu1-32, h+</i>	This study
147	<i>cdc10-V50, ura4-D18, h+</i>	Nurse 1211
370	<i>cdc25-22, his3-D1, leu1-32, ura4-D18, h+</i>	This study
374	<i>cdc25-22, ade6-M216, his3-D1, leu1-32, h+</i>	This study
381	<i>klp5Δ::ura4+, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
384	<i>klp5Δ::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
385	<i>klp5Δ::ura4+, cdc25-22, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
386	<i>klp6Δ::his3+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h-</i>	This study
389	<i>klp6Δ::his3+, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
390	<i>klp6Δ::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h-</i>	This study
393	<i>klp6Δ::ura4+, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
394	<i>klp5Δ::ura4+, klp6Δ::his3+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h-</i>	This study
397	<i>klp5Δ::ura4+, klp6Δ::his3+, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
398	<i>klp6Δ::his3+, cdc25-22, ade6-M210, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
399	<i>klp5Δ::ura4+, klp6Δ::his3+, cdc25-22, ade6-M210, his3-D1, leu1-32, ura4-D18, h+</i>	This study
474	<i>tea2Δ::his3+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h-</i>	H. Browning
485	<i>klp5::GFP::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
486	<i>klp6::GFP::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h-</i>	This study
487	<i>klp5Δ::ura4+, klp6::GFP::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
488	<i>klp6Δ::his3+, klp5::GFP::ura4+, ade6-M210, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
502	<i>klp5Δ::ura4+, cdc10-V50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
503	<i>klp6Δ::ura4+, cdc10-V50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
504	<i>klp5Δ::ura4+, klp6Δ::his3+, cdc10-V50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
505	<i>klp5Δ::ura4+, wee1-50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
506	<i>klp6Δ::ura4+, wee1-50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
507	<i>klp5Δ::ura4+, klp6Δ::his3+, wee1-50, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
508	<i>klp5Δ::ura4+, cdc2-33, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
509	<i>klp6Δ::ura4+, cdc2-33, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study
510	<i>klp5Δ::ura4+, klp6Δ::his3+, cdc2-33, ade6-M216, his3-D1, leu1-32, ura4-D-18, h+</i>	This study

mitosis; cytoplasmic microtubules are completely disassembled and a mitotic spindle forms within the nucleus. On completion of mitosis, interphase microtubules reappear, first as a “postanaphase array” around the new septum in G_1 and then as the paraxial bundles described above.

The *Schizosaccharomyces pombe* genome encodes nine kinesin-related genes from seven subfamilies and one cytoplasmic dynein (Hagan and Yanagida, 1990; Pidoux *et al.*, 1996; Yamamoto *et al.*, 1999; Brazer *et al.*, 2000; Browning *et al.*, 2000; Troxell *et al.*, 2001; *pombe* genome project). Among the kinesin subfamilies found in fission yeast, three have direct effects on microtubule dynamics and higher order microtubule organization (Goldstein and Philp, 1999). *S. pombe* expresses two members of the KAR3 subfamily, *pk11+* and *klp2+*. These promote microtubule disassembly and, together with dynein, are essential for meiosis (Troxell *et al.*, 2001). *S. pombe* also expresses *tea2+*, a member of the KIP2 subfamily, which promotes microtubule growth (Cottingham and Hoyt, 1997). Deletion of *tea2+* leads to unusually short microtubules and the mislocalization of the tip-specific marker Tea1p (Browning *et al.*, 2000). This compromises the polarity of *tea2Δ* cells, leading to their “T” shape. In budding yeast, the activity of Kip2p is antagonized by two different microtubule-destabilizing kinesins, Kar3p and Kip3p (Cot-

tingham and Hoyt, 1997; Huyett *et al.*, 1998; Miller *et al.*, 1998).

Here, we describe two fission yeast kinesins, *klp5+* and *klp6+*, that are both homologs to KIP3 kinesin in *Saccharomyces cerevisiae*. Our data demonstrate that these fission yeast kinesins influence the behavior of microtubules and cell morphology by fostering microtubule disassembly. They are also essential for meiosis.

MATERIALS AND METHODS

Strains and Cell Culture

All strains used in this study are listed in Table 1. The haploid strains 99 and 100 were generally used as wild type controls. Cell culture and genetic manipulations were performed with the use of standard techniques (Moreno *et al.*, 1991). Growth of *klpΔ* mutants was assayed on solid and liquid media over the range of temperatures commonly used to identify both cold and high temperature sensitivity in fission yeast (20, 25, 32, and 36°C). Growth rates were determined from cultures in yeast extract plus supplements (YES) medium during log phase with the use of the OD₅₉₅ at 20-min intervals. Temperature-sensitive mutants were incubated at 25°C for permissive temperature for growth, and at 36°C for restrictive growth. Cell transformations were carried out with the use of lith-

ium acetate/sorbitol (Moreno *et al.*, 1991) or polyethylene glycol (Elble, 1992)-based protocols. Strains were crossed on malt extract agar plates to induce meiosis, and the resultant spores were plated on YES medium to determine viability.

klp5⁺ and klp6⁺ Intron Mapping

The presence of small introns at the 5' end of both *klp5*⁺ and *klp6*⁺ was predicted from the DNA sequence provided by the *S. pombe* genome project and confirmed experimentally. The *klp5*⁺ intron was mapped by amplifying a cDNA clone from a *S. pombe* cDNA library (a generous gift of Drs. C. Norbury and B. Edgar, Imperial Cancer Research Fund, London, England) by polymerase chain reaction (PCR) with the use of primers KLP5-4 (gactaagaaatgtaacttgcaaatg) and KLP5A (ctgttgctgagtagcag). The *klp6*⁺ intron was confirmed by reverse transcriptase (RT) followed by PCR then a subsequent reaction of nested PCR, followed by DNA sequencing. RT-PCR was performed with reverse transcriptase and DNA polymerase following the manufacturer's specifications (Promega, Madison, WI). The substrate consisted of the poly(A⁺) fraction of RNA isolated from wild type cells with the use of standard methods (Sambrook and Russell, 2001). Parallel reactions were carried out with single primers, no added RNA, and no added RT, to control for primer artifacts or contaminating genomic DNA. RT-PCR first used primers KLP6-13 (cgatactgctatgaagaagggtc) and KLP6-9 (gcaaacacagtg-gcattatcc), followed with primers KLP6-13 and KLP6-14 (gatcaaat-gcatatgaacatc).

Computational Analysis of Sequences

Database searches were done with the BLAST algorithm (Altschul *et al.*, 1997). Protein sequences were aligned with Clustal W (Thompson *et al.*, 1994) then analyzed with the phylogenetic program PAUP, version 4.0, assuming maximum parsimony and with the use of a heuristic search method with stepwise addition (Sinauer Associates, Sunderland, MA). Coiled-coils predictions were done with the COILS program with the use of both MTK and MTIDK matrices, with and without the weighting option (Lupas *et al.*, 1991). The ProParam tools (www.expasy.ch/cgi-bin./protparam) were used to determine molecular weights and theoretical isoelectric points (pI).

Construction of Null Strains

The *klp5*⁺ and *klp6*⁺ null mutant strains were constructed with the use of a single-step gene replacement protocol and the selectable markers *his3*⁺ (Ohi *et al.*, 1996) or *ura4*⁺ (Grimm *et al.*, 1988) in the appropriate auxotrophic backgrounds (Table 1).

The *klp5*-null allele was constructed with the use of long flanking regions of *klp5*⁺ to target integration. The 5'-flanking 575 base pairs were PCR amplified with primers KLP-L1 (atagcgctcactagtctct) and KLP-L2 (gccagtgggattgttagctaaagagcgagaacgcgt), which includes sequence overlapping the 5' end of *ura4*⁺. The 3'-flanking 520 base pairs were PCR amplified with primers KLP-L3 (gcgtttgtt-tcttaggcgaagctcacagcttgatcaactgctg), which contains sequence overlapping the 3'-end of *ura4*⁺, and KLP-L4 (tgtacattggaggtggcaga). The resulting PCR products were then used as long primers for a second PCR reaction, with the *ura4*⁺ gene as the substrate and the resulting product used in transformations.

The *klp6*-null allele was constructed with the use of long flanking PCR primers directly. The nutritional marker genes were PCR amplified with primers containing 75 bases identical to the 5'- and 3'-flanking regions *klp6*⁺. The primers were KLP6-URA4-5' (caagcttc ccatactgtgttcttcttaataagcttcacacaaactaaacaaat tcatctctagaatcagattatagcagataccactggcctatattgtat gcatgtt) with KLP6-URA4-3' (gaagaaaagccaatgaggagt tgaattgttcttccaaaaaattatataccaactatttgattga aaaccgatctcgttggtttccaaacccaatgtttataaccaag) and KLP6-HIS3-5' (caagcttccatactgtgttcttcttaataag

cttcacacaaactaaacaaattcattcctagaatcagattatagc atactgctttggaaatgaaagacatattggagc) and KLP6-HIS3/3' (gaagaaaagccaatgaggagtgtgattgttcttccaaaaa aattataccaactatttgattgagtaaaaccgatctcgttggcagcg gttataatctttaaattagcg).

Diploid cells constructed from strains 99 and 100 (Table 1) were transformed with the PCR products, and Ura⁺ or His⁺ transformants were selected for growth on defined medium. Tetrad analysis showed that all four colonies in each tetrad were viable, whereas the marker genes segregated 2:2, indicating a single integration in a nonessential locus.

Homologous integration at the *klp5*⁺ locus was confirmed by PCR with the use of primers KLP5-1 (gactcaccaacattcatcctcaac) with URA4-1 (caagatagaatggatgtttgaaataaacg) or URA4-E (catgctctcaacattaccac). Homologous integration at the *klp6*⁺ locus was confirmed by PCR with the use of primers KLP6-1 (cgactatggctcatagata-cattggatgt) and HIS3-3 (ctaattgcgcttgcttcc) or URA4-E.

The *klp5Δ*, *klp6Δ*, and *klp5Δ klp6Δ* double mutants are collectively referred to as "*klpΔ*" when either the same experiment was done with all three strains or the same general conclusion is being drawn from experiments done with all three strains.

Construction of GFP-tagged Strains

A plasmid vector was constructed containing a [Glycine-Alanine]₂ spacer, three tandem copies of the Pk1 epitope (Southern *et al.*, 1991), the green fluorescent protein (GFP) (F64L, S65T allele) (Cormack *et al.*, 1996), the *nmt1*⁺ terminator sequence (Maundrell, 1993), and the *ura4*⁺ gene. A DNA molecule containing this construction of (GA)₂-(Pk1)₃-GFP-*ura4*⁺ was PCR amplified with the use of a 5' primer containing 75 bases identical to the 3' end of each respective Klp open-reading frame and 33 bases, in-frame, containing the Glycine-Alanine spacer and the [Pk1]₃ sequence. The 3' primers consisted of 81 bases identical to the 3' end of each klp, starting six base pairs 3' of the stop codon, and 25 bases corresponding to the 3' end of the *ura4*⁺ gene. The Klp-Pk1-GFP-*ura4*⁺ PCR product was used to transform diploids and Ura⁺ integrants identified. Homologous integration was confirmed by PCR with primers to the Klp sequence, GFP, and *ura4*⁺. The Klp-Pk1-GFP region from the selected integrant strains were PCR amplified and sequenced to confirm in-frame integration and to identify any PCR-generated sequence errors in the constructs. One missense mutation was found in the GFP portion of Klp5pGFP (agt-ggt; S2G) and one in the GFP portion of Klp6pGFP (ctt-ctc; L40P), but neither of these changes had a noticeable effect of the fluorescence properties of the GFP fusions.

The *klp5*-GFP-*ura4*⁺ fragment was amplified with primers 5'KLP5GFP (cttcacatctttcaaatccagctaacattattaggaaatc ttttaagcatggctgaaaacgaagaagagaagccaccggaggag agctcatgggtattcctaaccctttg) and 3'KLP5GFP (catatca tcaagccttatccgttttttttttttaataatacccaacaggatattta gaggattctgtatttgaatatacgggttccaaacacaaatgtttata accaag). The *klp6*-GFP-*ura4*⁺ fragment was amplified with primers 5'KLP6GFP (caaccagtagcgcgtatatacgttcttcacaaaccttt acaaaaaactggcgggactgagaatactcctaattgctggaggag agctcatgggtattcctaaccctttg) and 3'KLP6GFP (gaagaa aagccaatgaggagtgtgattgttcttccaaaaaaattatacca actatttgagtgaanaaccgatctcgttgggttccaaacacaaatgt ttaataaccaag). The italicized sequences are from the *klps*, and the remaining are from Gly-Ala/Pk1 (5') or *ura4*⁺ (3') sequence. The *klp5*-GFP integration was confirmed with primers KLP5-1 (gactcac-caacattcatcctcaac) with GFP-HVEM3 (gtacataaccttcgggcatg) and KLP5-L4 (gtacattggaggtggcagac) with URA4-E. The *klp6*-GFP integration was confirmed with primers KLP6-3 (ctcattctccaaatggc-caac) with GFP-HVEM3 and KLP6-1 with URA4-E.

Microscopy

Cells were grown to mid log phase (1–3 × 10⁵ cells/ml) for microscopy. Experiments with GFP-tagged genes were generally done at

A) KIP3 family **B) Domain map** **C) amino-terminal alignment**

Klp6p- GSSISVAVRVRPFTEREK
 Klp5p- QSSITVTVRVRPFSTAES
 Kip3p- QSSIVVAIRVRPFSTMEK

D) Coils prediction (neck) **E) Tail boxes**

Klp5p- EGATMYADVLQDEVDLLKSIENQVLDA.....KKRVRF
 Klp6p- EGADMYADMLQDDVDLLKSIENQILDA.....KKRVRF

Figure 1. Sequence alignments and structural features of the KIP3 subfamily of kinesins. (A) Bootstrap analysis produced a phylogenetic tree made with *S. pombe* Klp5p and Klp6p, *S. cerevisiae* Kip3p and Kip2p, *Drosophila melanogaster* KLP67A, and *Caenorhabditis elegans* LF224KLP (see MATERIALS AND METHODS). The numbers represent bootstrap values for 100 replicas. (B) Generalized domain map for the KIP3 subfamily. The amino acid that marks the approximate boundary between each domain is marked on the scale below the diagram. See below for the exact positions. (C) *BESTFIT* alignment of the amino-terminal leader portion of KIP3 kinesins. Identical amino acids are boxed in dark gray, and similar amino acids in light gray (beginning at amino acid 3). (D) *COILS* (Lupas *et al.*, 1991) prediction for Klp5p with the use of a window of 28. A coiled-coil probability of 1.0 is calculated for Klp5p amino acids 396–436, corresponding to the “neck” in B. A similar graph is generated for

25°C, and others were done at the temperatures noted. Fixed cells were prepared for tubulin and DNA staining with the use of a double aldehyde method (Hagan and Hyams, 1988), and antibodies were applied as previously described (West *et al.*, 1998). DNA staining was also done with cells fixed by adding 1/10 volume of cell culture to methanol at –20°C for 2–15 min. Cells were collected by centrifugation, resuspended in phosphate-buffered saline (pH 7.4) containing 1 µg/ml 4',6-diamino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) and mounted on glass slides.

Live cells were analyzed by visualizing microtubules with GFP-α-tubulin, Klp proteins with their GFP fusions, and DNA with Hoescht 33342 (Sigma). The GFP-α-tubulin plasmid pDQ105 (Ding *et al.*, 1998) was transformed into cells and the resultant strains grown in defined medium (Moreno *et al.*, 1991) containing 5 µg/ml thiamine (Sigma) to limit levels of expression (Maudrell, 1993) of the GFP-α-tubulin. Cells were collected by centrifugation (5–10 ml) at 3000 rpm for 3 min in a Beckman Coulter CS-6 centrifuge, and resuspended in 5 ml of YES, pH 7.5, containing 2 µg/ml Hoescht 33342 for ~30 min. Cells were then mounted on glass coverslips, and images were collected on a Zeiss Axiophot2 fluorescence microscope with a 100× Plan-APO objective lens (numerical aperture 1.4). Images were captured with a Cooke SensiCam charge-coupled device camera and processed with the use of the SlideBook software package (Intelligent Imaging Innovations, Denver, CO) as follows. Generally, 6 to 12 focal planes spaced at 200- or 300-nm intervals along the Z-axis were collected, with 2 × 2 binning (voxel size 67 nm), with the use of exposure times of ~300 ms in the DAPI channel (Hoescht33342 or DAPI) and 500–1000 ms in the fluorescein isothiocyanate channel (GFP) per focal plane. The images were deconvolved with the use of either a No Neighbor (short Z-series) or Nearest Neighbor (long Z-series) algorithm, with a subtraction factor of 0.6–0.9. A two-dimensional image was then rendered with the use of an orthographic projection of pixel maxima along lines parallel to the optical axis. The resulting image was prepared as figures with the use of CorelDraw (Ottawa, Ont).

RESULTS***klp5+*, *klp6+*, and KIP3 Constitute a Kinesin Subfamily**

We have characterized two of the nine kinesins of fission yeast, designated *klp5+* (kinesin-like protein) (accession no. Z97211; right arm of chromosome 2, centromere proximal) and *klp6+* (accession no. ALO23587; left arm of chromosome 2, telomere proximal). Several different sequence alignment algorithms (see MATERIALS AND METHODS) indicated that Klp5p and Klp6p are more closely related to each other than to any other proteins, and each is equally similar to the *S. cerevisiae* motor KIP3. For example, an alignment of Klp5p, Klp6p, Kip3p and several other kinesins with the use of the phylogenetic program PAUP (version 4.0), groups *klp5+* and *klp6+* together with KIP3 but separates them from other kinesins (Figure 1A). Although these two kinesins are not genetically linked, they are physically linked on the same chromosome and contain short introns that are identically placed at their 5' ends, as confirmed by PCR and DNA sequencing (see MATERIALS AND METHODS). The *klp5+* intron splits the 11th codon (g/gtagt...aaatag/tc) and is 55

Klp6p, with a probability of 0.91 for amino acids 404–440 (our unpublished data). (E) *BESTFIT* alignment of the “tail” boxes for Klp5p and Klp6p. The position of these amino acids is indicated as boxes in the tail of B (each box begins at Klp5p, amino acid 554, then 691; Klp6p, amino acid 548, then 671).

base pairs in length. The *klp6*⁺ intron also splits the 11th codon (g/gtaaga...atcaag/tt) and is 39 base pairs in length.

Sequence analyses of the predicted proteins identified distinguishing motifs and generated a models their domain structures (Figure 1B). Klp5p is predicted to contain 883 amino acids and a molecular mass of 99 kDa, whereas Klp6p is 784 amino acids (aa) with a predicted molecular mass of 88 kDa. As with many other kinesins, the similarity seen is largely confined to ~320 amino acids of the motor domain. The motor domains of Klp5p and Klp6p are 66% identical/76% similar, whereas those of both Klp5p and Klp6p are 62% identical/73% similar with Kip3p.

The motor domain of each polypeptide is located near its amino-terminal end, but there is a short nonmotor sequence at the amino terminus (Figure 1B). This amino-terminal region is variable in size (Klp5p, ~90 aa; Klp6p, ~110 aa) and is unique to each protein, with the exception of a conserved sequence of 18 aa (Figure 1C). A similar sequence is also found in *UNC-104* kinesin subfamily members (for Internet attachments to sequence files see the Kinesin Home Page; <http://www.blocks.fhcrc.org/%7Ekinesin>), but the significance of this similarity is unknown.

Immediately carboxy terminal from the motor domain, these kinesins are predicted to have a single turn of coiled-coil (see MATERIALS AND METHODS) (Figure 1D). The position, length, and sequence of this region are analogous to the $\alpha 7$ coil of kinesin heavy chain, which has been shown to be sufficient for dimerization and critical in specifying plus-end directed motility (reviewed in Sack *et al.*, 1999).

The carboxy-terminal half of each of these kinesins constitutes the tail domain, but there are no significant structural predictions for this domain. Each tail is also of variable length (Klp5p, ~450 aa; Klp6p, ~360 aa) and its sequence is unique to each protein. The theoretical isoelectric points for the tails of Klp5p (pI = 5.6) and Klp6p (pI = 10.6) further exemplify their differences. There is, however, a short region of 34 amino acids, designated the "tail box," which is highly conserved among just these two fission yeast Klps (Figure 1E). This sequence does not show any significant similarity with other proteins, although the lysine rich region bares some resemblance to several unrelated DNA binding proteins.

***klp5*⁺ and *klp6*⁺ Are not Essential for Vegetative Growth**

The functions of *klp5*⁺ and *klp6*⁺ were investigated through the construction of null mutants. The entire open-reading frame for each kinesin was deleted, and the resultant strains, designated *klp5* Δ and *klp6* Δ , were analyzed for growth under various conditions (see MATERIALS AND METHODS). The growth of *klp5* Δ and *klp6* Δ strains on solid medium was virtually indistinguishable from wild type (Figure 2). They were, however, slightly darker on plates containing the exclusion stain Phloxin B (Sigma), indicating a subtle loss of viability (our unpublished data). The lack of a significant growth defect in these *klp* Δ strains was confirmed by examining growth in liquid culture. The *klp6* Δ strain did exhibit a slightly longer doubling time at both moderate and high temperatures (Table 2). Surprisingly, the *klp5* Δ *klp6* Δ double mutant was also viable and not observably compromised (Figure 2 and Table 2). Rather, growth of *klp5* Δ *klp6* Δ more closely resembled that of the *klp5* Δ single mutant, so the

Figure 2. The *klp5* Δ , *klp6* Δ , and *klp5* Δ *klp6* Δ mutants are viable. Strains were grown in liquid culture to early log phase then plated as fivefold serial dilutions onto agar and grown at 32°C for 3 d.

klp5⁺ deletion is epistatic to the *klp6*⁺ deletion. Throughout our analyses of these two kinesin genes, we repeatedly observed the strongest phenotype in the *klp6* Δ strain, although the phenotypic differences among the *klp* Δ mutants are subtle.

***klp5* Δ and *klp6* Δ Cells Have Long Microtubules**

Several kinesins alter the dynamics of microtubules in cells, either by increasing or decreasing tubulin assembly (reviewed in Hunter and Wordeman, 2000). The loss of a motor enzyme with either of these activities can have a distinct effect on cells, and it can therefore be studied in several ways, including changes in cell morphology and microtubule organization, interactions with tubulin mutants, and altered sensitivity to microtubule drugs. We have used all these assays to probe microtubule behavior in the *klp* Δ alleles and found that the microtubules are stabilized by the absence of either Klp5p or Klp6p.

Genetic interactions between *klp* Δ and tubulin were examined by constructing double and triple mutants between *klp* Δ strains and mutants in both α - and β -tubulin. The *klp* Δ mutants were synthetically lethal with the cold-sensitive, thiabendazole (TBZ)-sensitive, α -tubulin mutant *nda2-K52* (Toda *et al.*, 1984), because double and triple mutants were not identified at the permissive (32°C) or restrictive (20°C) temperatures for *nda2-K53*. No interaction was apparent between the cold-sensitive, TBZ-resistant, β -tubulin mutant *nda3-311* (Hiraoka *et al.*, 1984), and the *klp* Δ strains (our unpublished data).

Table 2. Growth rates for the *klp* Δ mutants

Cultures were grown in YES medium from early to late log phase and the OD₅₉₅ recorded at 20-min intervals. Doubling times were derived from three independent experiments, and the variations are given as the standard error of the mean.

Strain	Doubling time (h)	
	25°C	35.5°C
Wild type	3.3 $\pm$ 0.1	2.2 $\pm$ 0.1
<i>klp5</i> Δ	3.4 $\pm$ 0.1	2.2 $\pm$ 0.1
<i>klp6</i> Δ	3.6 $\pm$ 0.1	2.3 $\pm$ 0.1
<i>klp5</i> Δ , <i>klp6</i> Δ	3.4 $\pm$ 0.1	2.2 $\pm$ 0.1

Figure 3. *klpΔ* mutants are resistant to the microtubule-destabilizing drug TBZ. Strains were grown in liquid medium to early log phase then plated in fivefold serial dilutions onto agar plates containing the indicated concentration of TBZ and incubated at 32°C for 3 d.

The *klpΔ* mutants grew comparatively well in the presence of the microtubule-destabilizing drug TBZ, as demonstrated by plating assays (Figure 3). Wild-type cells failed to grow in TBZ concentrations higher than 25 μg/ml at 32°C, but *klpΔ* strains continued to grow in 75 μg/ml. Comparable differences in resistance to TBZ among wild type and *klpΔ* strains were also seen at 25 and 36°C, although the lower temperature increased the sensitivity of all strains to TBZ. No differences in TBZ resistance were observed among the three *klpΔ* strains. The ability of the *klpΔ* strains to withstand elevated concentrations of a microtubule poison suggests that microtubules are more stable in the absence of either of these two kinesins than in wild type cells.

If the microtubules in *klpΔ* cells are indeed hyperstable then their length or organization would likely be altered. To test this possibility, the microtubules in living cells were examined directly with the use of GFP- α -tubulin. Wild-type

interphase cells contain a modest array of straight microtubules, which generally run essentially from one end of the cell to the other (Figure 4A; reviewed in Hagan, 1998). The *klpΔ* strains, on the other hand, frequently contained microtubules of sufficient length to curl around the ends of the cells (Figure 4, B–D, arrows). As described for TBZ resistance, differences between wild type and *klpΔ* were apparent, but no significant difference in microtubule length or organization was observed among the *klpΔ* strains. The tendency for *klpΔ* microtubules to grow to the ends of cells and bend, together with the resistance of these strains to TBZ, is consistent with the hypothesis that both Klp5p and Klp6p promote microtubule disassembly.

cdc25⁺ Function Modulates Microtubule Behavior in the Absence of klp5⁺ and klp6⁺

Although defects in cytoplasmic microtubules often result in defects in cell shape, the *klpΔ* cells have an apparently normal cell morphology, both during log phase growth and as they emerge from stationary phase (our unpublished data). However, abnormal cell morphology was observed in certain genetic backgrounds that significantly increase cell size. The strongest effect was seen with the temperature-sensitive cell cycle progression mutant *cdc25-22* (Figure 5). At restrictive temperature this allele arrests cell cycle progression at the G₂/M boundary, resulting in cells that are >3 times longer than wild type (Fantès, 1979). Even at permissive temperature cell cycle progression is somewhat delayed, resulting in cells that are ~50% longer than wild type. The *cdc25-22* cells grow with a straight, symmetric shape at both permissive and restrictive temperatures (Figure 5, A and B). The *klpΔ, cdc25-22* strains, on the other hand, become progressively bent as they get longer at restrictive temperature, forming “C”-shaped cells (compare Figure 5, B and D). After 8 h of arrest, virtually all of the cells had an abnormal morphology. Although the bending was pronounced, it was generally symmetric, and the cells septate in the middle of the C after they were released from G₂ arrest and divided (our unpublished data).

The organization of the microtubule cytoskeleton in these cells was determined with GFP- α -tubulin. The *cdc25-22* cells had long, straight microtubules at both permissive and restrictive temperature, although the microtubules often failed to reach the ends of the longer cells (Figure 5, E and F). The *klpΔ, cdc25-22* cells, in contrast, had especially long microtubules that wrapped around the ends of the cells (compare Figure 5, E and G), even at permissive temperature. At restrictive temperature, these microtubules sometimes curled before they reached the ends of the cells (Figure 5H,

Figure 4. *klpΔ* mutants strains have longer, bent microtubules. Microtubules were visualized with GFP- α -tubulin by deconvolution microscopy (see MATERIALS AND METHODS). (A) Wild-type cells with straight microtubules. (B) *klp5Δ* cells with long, curved microtubules at the ends (arrow). (C) *klp6Δ* cells with long, curled microtubules (arrow). (D) *klp5Δ klp6Δ* cells are similar to the single mutants. Bars, 5 μm.

Figure 5. Cell cycle arrest mutant *cdc25-22* has a bent-cell shape and particularly long, bent microtubules in the *klpΔ* backgrounds. (A–D) Differential interference contrast images of *cdc25-22* and *cdc25-22, klp5Δ* cells. (A) *cdc25-22* cells at permissive temperature. (B) *cdc25-22* cells at restrictive temperature for 6 h. (C) *cdc25-22, klp5Δ* cells at permissive temperature. (D) *cdc25-22, klp5Δ* cells at restrictive temperature for 6 h. (E–H) Microtubules were visualized with GFP- α -tubulin by deconvolution microscopy. (E) *cdc25-22* cells at permissive temperature. Inset is a higher magnification of the tip of one cell (arrow) with straight microtubules. (F) *cdc25-22* cells at restrictive temperature for 6 h. (G) *klp5Δ cdc25-22* cells at permissive temperature. Inset is a higher magnification of the tip of one cell (arrow) with microtubules curled around the end. (H) *klp5Δ cdc25-22* cells at restrictive temperature for 6 h. Curled microtubules are present but not always at the end of the cell (arrows). Bars, 5 μ m.

arrows). This phenotype was the most pronounced in the *klp5Δ, klp6Δ, cdc25-22* triple mutants.

The extreme elongation of microtubules in these cells could be due either to a general effect from increased length, or to a more direct, regulatory interaction between the *klp5⁺* and *klp6⁺* genes and *cdc25⁺*. To distinguish between these possibilities, we constructed *klpΔ* strains that grew longer than wild type as a result of factors independent of *cdc25⁺*, and we also examined interactions between *klpΔ* and genes directly related to the function of *cdc25⁺*.

The *cdc10-V50* mutant arrests cell cycle progression at START in G₁ at 36°C, but the cells continue to elongate at one end, becoming long and straight, similar to *cdc25-22* (Marks *et al.*, 1992). The microtubule arrays in *cdc10-V50* grown at either permissive or restrictive temperature did not look different from wild type. The *klpΔ, cdc10-V50* cells, on the other hand, became bent at restrictive temperature (Figure 6, A and B). These cells were similar to the *klpΔ cdc25-22* mutants, except they were generally bent at just one end, forming “J-” rather than C-shaped cells. The microtubules in *klpΔ, cdc10-V50* cells sometimes bundled, but they were not long and curled as in *cdc25-22* (Figure 6, C and D).

Fission yeast diploids cells are ~85% longer than haploids (Nurse and Thuriaux, 1980), and they have microtubule arrays similar to those seen in haploid cells (reviewed in Hagan, 1998). The *klpΔ/klpΔ* homozygous diploid cells were appropriately long and slightly bent, with an occasional curled microtubule at 25°C, but at 36°C these effects became pronounced (Figure 6, E–H).

Together, these results indicate that the normal shape of fission yeast cells is disrupted when either *klp5⁺* or *klp6⁺* is deleted and the cells are sufficiently long. This effect is enhanced by growth at elevated temperature. The effect of *klpΔ* on microtubule structure varies depending on the genetic background, with the greatest effect observed in *cdc25-22* cells.

We asked whether *cdc25⁺* might play a more direct role in microtubule/motor regulation by generating double mutants with *klpΔ* and other genes related to *cdc25⁺* function. The *wee1⁺* kinase acts antagonistically to the *cdc25⁺* phosphatase, inhibiting the cyclin-dependent kinase *cdc2⁺*, and thus restraining entry into mitosis. The lose-of-function allele, *wee1-50*, produces cells that are small (Fantès, 1981; reviewed in Forsburg and Nurse, 1991) but have wild type microtubule arrays at both permissive and restrictive temperature (Figure 6, I and J). The *klpΔ, wee1-50* mutants showed no obvious shape abnormality at permissive temperature, but at restrictive temperature they were 15% shorter than *wee1-50* alone at the same temperature. At both temperatures, the microtubules in *klpΔ, wee1-50* cells tended to curl at the ends of the cells (Figure 6, K and L).

Both Cdc25p and Wee1p act on the kinase encoded by *cdc2⁺*, the master regulator of mitosis (reviewed in Forsburg and Nurse, 1991). We therefore looked for an effect of the temperature-sensitive allele *cdc2-33* on cell morphology and/or microtubule organization in the *klpΔ* backgrounds. Both cell morphology and microtubule organization in the *cdc2-33* parental strain resembled that of *cdc25-22*, and they also elongated at restrictive temperature (our unpublished data). The *klpΔ, cdc2-33* cells did tend to bend, but their microtubules did not curl more than was seen in wild type cells (Figure 6, M–P).

Figure 6. Cell morphology and microtubule organization in cell cycle mutants and diploids. (A) Bright field image of *klp5Δ, cdc10-V50* cells at permissive temperature. (B) Bright field image of *klp5Δ, cdc10-V50* cells at restrictive temperature for 6 h. (C) Microtubule labeling in *klp5Δ cdc10-V50* cells at permissive temperature. (D) Microtubule staining in *klp5Δ cdc10-V50* cells at restrictive temperature for 6 h. (E) Bright field image of *klp5Δ/klp5Δ* diploid cells at 25°C. (F) Bright field image of *klp5Δ/klp5Δ* diploid cells at 36°C. (G) Microtubule labeling in *klp5Δ/klp5Δ* diploid cells at 25°C. (H) Microtubule staining in *klp5Δ/klp5Δ* diploid cells at 36°C. (I–L) Microtubule labeling in *wee1-50* backgrounds: *wee1-50* cells at permissive temperature (I); *wee1-50* cells at restrictive temperature for 6 h (J); *klp5Δ wee1-50* cells at permissive temperature (K); *klp5Δ wee1-50* cells at restrictive temperature for 6 h (L). (M) Bright field image of *klp5Δ, cdc2-33* cells at permissive temperature. (N) Bright field image of *klp5Δ, cdc2-33* cells at restrictive temperature for 4 h. (O) Microtubule labeling of *klp5Δ, cdc2-33* cells at permissive temperature. (P) Microtubule labeling of *klp5Δ, cdc2-33* cells at restrictive temperature for 4 h. Bars, 10 μ m.

These experiments reveal that *cdc25⁺* can modulate microtubule behavior in a way that is normally masked by the expression of Klp5p and Klp6p, suggesting that this phosphatase may act on microtubule proteins to modulate tubulin dynamics.

Klp5p and Klp6p Localize to Cytoplasmic Microtubules and Mitotic Spindles

To better understand the functions of Klp5p and Klp6p in microtubule organization, each of these proteins has been

Figure 7. Klp5p and Klp6p localize to cytoplasmic microtubules in interphase and spindles in mitosis. Klp5-GFP and Klp6-GFP were visualized in live cells (green) with DNA staining (Hoescht 33342, blue) by deconvolution microscopy. (A) Klp5p-GFP localized to cytoplasmic microtubules in interphase. (B) Klp6p-GFP localized to cytoplasmic microtubules in interphase. (C) Klp5p-GFP localized to a mitotic spindle in the nucleus, and to the “astral” microtubules in the cytoplasm (arrows). (D) Klp6p-GFP localized to a mitotic spindle in the nucleus. (E) Klp5p-GFP redistributes to the interphase microtubules as they are formed at the conclusion of mitosis. (F) Klp6p-GFP redistributes to interphase microtubules after mitosis. These first appear as the “post anaphase array” near the region of septation. Bar, 5 μ m.

localized in vivo, with the use of GFP fused to the carboxy terminus by integration of appropriate sequences at the *klp5*⁺ and *klp6*⁺ loci. This strategy assures that expression of each chimeric protein is under the control of its endogenous promoter (see MATERIALS AND METHODS). The Klp5p-GFP and Klp6p-GFP strains showed normal growth on plates and produced viable spores in meiotic crosses, indicating that the fusions did not significantly compromise motor function. Live cells were examined through the cell cycle, and the same pattern of localization was observed for both Klp5p and Klp6p.

The Klp-GFP proteins localized to cytoplasmic microtubules in interphase cells, with no detectable preference for a subset of microtubules or bias toward one microtubule end or the other (Figure 7, A and B). As the cells entered mitosis, the Klp-GFP proteins localized to the mitotic spindle and the astral microtubules (Figure 7, C and D). Immediately upon exit from mitosis, the Klp-GFP proteins relocated to cytoplasmic microtubules, initially to the postanaphase array, and subsequently to the normal interphase microtubules (Figure 7).

To determine whether the localization of either Klp5p or Klp6p is dependent on expression of the other, we crossed each Klp-GFP strain into the reciprocal *klp* Δ strain and examined the distribution of fluorescence. Both of these double mutant strains showed the same localization of Klp5p and Klp6p in their reciprocal *klp* Δ background as was seen in the wild type background (our unpublished data).

Together, these data demonstrate that both Klp5p and Klp6p localize to cytoplasmic microtubules in interphase and to both the mitotic spindle and astral microtubules in mitosis. The localizations of Klp5p and Klp6p were indistinguishable, although each protein localized independently of the other.

Interactions with Other Kinesins

Complex interactions among kinesin subfamily members have been described in several systems (reviewed in Goldstein and Philp, 1999). Of particular interest for our studies of *klp5*⁺ and *klp6*⁺ are the antagonistic interactions between budding yeast *KIP3* and *KIP2* (Cottingham and Hoyt, 1997; Miller *et al.*, 1998), and the aspects of overlapping function between *KIP3* and *KAR3* (DeZwaan *et al.*, 1997; Cottingham *et al.*, 1999). Similar interactions in fission yeast have been investigated by constructing double, triple, and quadruple mutants between the *klp* Δ strains and the relevant fission yeast homologs.

The *KIP2* homolog *tea2*⁺ has previously been characterized; null mutants are viable but have short microtubules and often take on a “T” morphology (Browning *et al.*, 2000). The *klp* Δ , *tea2* Δ double and triple mutants were as viable as the parental single and double mutant strains. Further, the T morphology phenotype described for *tea2* Δ cells was neither enhanced nor rescued in *klp* Δ backgrounds (our unpublished data). These data suggest a lack of significant functional interaction among these kinesins in fission yeast.

S. pombe has two *KAR3* homologs, *pk11*⁺ and *klp2*⁺ (Pidoux *et al.*, 1996; Troxell *et al.*, 2001). Neither of these kinesins is essential, either individually or together, but null mutants have altered sensitivities to TBZ and severe meiotic phenotypes. The four possible triple mutant combinations, and the *klp5* Δ , *klp6* Δ , *pk11* Δ , *klp2* Δ quadruple mutant were constructed and analyzed for growth. There were no obvious growth defects in any of the triple mutants, and the quadruple mutant showed only a slight growth defect at 36°C (our unpublished data). These data suggest a lack of significant functional redundancy among the members of the *KIP3* and *KAR3* kinesin subfamilies in fission yeast.

klp5⁺ and *klp6*⁺ Are Essential for Meiosis

Kinesins (Meluh and Rose, 1990) and dynein (Yamamoto *et al.*, 1999; Troxell *et al.*, 2001) are important for meiosis in

Figure 8. *klpΔ* strains have defects in meiosis as indicated by the number and morphology of spores in meiotic asci. (A) Differential interference contrast image of normal asci from a homozygous wild type cross, each contains four symmetric spores. (B) Ascus from a *klp5Δ* homozygous cross with a single, large spore (arrow). (C) Ascus from a *klp6Δ* homozygous cross with several small spores (arrowheads) and incompletely fused (arrow) mating cells. Bar, 5 μ m.

several organisms, including yeasts, so we asked whether *klp5⁺* and *klp6⁺* play a role in meiosis of fission yeast. *S. pombe* cells initiate a meiotic life cycle when they are starved for nitrogen and when haploid cells of opposite mating types are present. The meiotic cycle culminates with the formation of an ascus containing four symmetric, haploid spores (Figure 8A). This configuration was observed in 90% of homozygous wild type zygotic asci. However, the *klp5Δ* and *klp6Δ* strains showed only ~3% wild type asci (four symmetric spores) ($n = \sim 1000$) in crosses homozygous for either *klpΔ* allele. Heterozygous crosses between these *klpΔ* alleles and wild type yielded a lower frequency of abnormal asci (30–60% normal; $n = 300$). A wide range of morphological anomalies was seen in *klpΔ* asci, but the occurrence of asci that contained one single, large spore in *klp5Δ* crosses

suggests a defect in the first meiotic division (Figure 8B). The *klp6Δ* homozygous crosses produced some one-spore asci, but pinched asci were also seen, which may represent a failure to complete conjugation (Figure 8C).

Spore viability was greatly reduced in *klpΔ* homozygous crosses, whereas heterozygous crosses of either *klp5Δ* or *klp6Δ* with wild type showed nearly wild type spore viability (Table 3A). In heterozygous crosses between *klp5Δ* and *klp6Δ*, spore viability was intermediate between that observed for wild type and homozygous *klpΔ* crosses (Table 3A). Equivalent results were obtained when the parental strains were either heterozygous for *klp5⁺* and *klp6⁺* in *cis* (*klp5Δ klp6Δ* $\times$ wt) or *trans* (*klp5Δ* $\times$ *klp6Δ*).

To distinguish between defects in the initial formation of a diploid zygote versus subsequent meiotic DNA segregation, stable *klpΔ* diploid strains were isolated, grown vegetatively for several generations, and then induced to sporulate. The relative frequency of stable diploids isolated from *klp5Δ* crosses was not significantly different than from wild-type crosses, but *klp6Δ/klp6Δ* diploids were less frequently found. These results suggest that zygote formation is not significantly affected in *klp5Δ* but is compromised in *klp6Δ*. Spore viability was not increased in any of the *klpΔ* strains by first selecting for stable diploid cells, indicating that meiotic defects subsequent to karyogamy result in loss of spore viability (Table 3B).

DISCUSSION

KIP3 Kinesin Subfamily

We propose a new subfamily of kinesin-like proteins that includes the fission yeast genes *klp5⁺* and *klp6⁺* and the group's founding member, *KIP3*, from *S. cerevisiae*. There are several shared characteristics that define members of this new kinesin subfamily. First and foremost, there is considerable sequence similarity in their motor domains and in their general domain structure and organization (Figure 1). Kip3p, Klp5p, and Klp6p also all localize to cytoplasmic and spindle microtubules (Figure 7) (DeZwaan *et al.*, 1997). Furthermore, Klp5p and Klp6p share with Kip3p an activity that fosters microtubule disassembly, as evidenced by the unusually long and/or robust microtubules found in null mutants (Figures 3–6) (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997; Miller *et al.*, 1998). Microtubule-disassembling activity has also been described for the KinI kinesin subfamily (XKCM1, Walczak *et al.*, 1996; MCAK, Maney *et al.*, 1998; Desai *et al.*, 1999) and for Kar3p (Endow *et al.*, 1994). There is, however, no obvious sequence similarity between members of the KIP3 subfamily and the other kinesins with “exotubulase” activity. It is possible that each kinesin subfamily uses a different mechanism to promote microtubule disassembly or that there is conservation in protein structure that is not apparent from the amino acid sequence.

Although all the KIP3 family members share the feature of promoting microtubule disassembly, their roles in the physiology of each yeast cell are distinct in several important ways. First, the *klpΔ* mutants of fission yeast show disrupted patterns of DNA segregation along the mitotic spindle but nuclear positioning appears normal (West *et al.*, 2001). *KIP3*, on the other hand, is part of a pathway required for proper nuclear migration to the bud neck early in mitosis, but

Table 3. *klpΔ* spore viability

(A) Spore viability from zygotic asci. Random spores from each cross were isolated, plated on YES medium, and colonies counted after 5 d. (B) Spore viability for azygotic asci. Stable diploids were isolated from each indicated cross, grown vegetatively to form colonies, and then induced to sporulate (see MATERIALS AND METHODS). Spore viability was then determined as described for zygotic crosses. Spores (1000) were plated for each cross.

(A) Spore viability—zygotic asci

Strain	Spore viability			
	Wild type	<i>klp5Δ</i>	<i>klp6Δ</i>	<i>klp5Δ, klp6Δ</i>
Wild type	73%			
<i>klp5Δ</i>	74%	1%		
<i>klp6Δ</i>	60%	28%	1%	
<i>klp5Δ, klp6Δ</i>	25%	1%	5%	2%

(B) Spore viability—azygotic asci

Strain	Spore viability			
	Wild type	<i>klp5Δ</i>	<i>klp6Δ</i>	<i>klp5Δ, klp6Δ</i>
Wild type	81%			
<i>klp5Δ</i>	60%	2%		
<i>klp6Δ</i>	60%	45%	3%	
<i>klp5Δ, klp6Δ</i>	25%	9%	2%	1%

movement of the chromosomes along the spindle appears normal (DeZwaan *et al.*, 1997). Second, *klp5⁺* and *klp6⁺* are not essential for vegetative growth, even in the absence of KAR3 family members *pk11⁺* and *klp2⁺*. KIP3 is likewise nonessential, but either KIP3 or KAR3 (Meluh and Rose, 1990) (together with at least one BimC family member) must be present for viability, suggesting that budding yeast requires at least one microtubule-destabilizing motor protein for survival (DeZwaan *et al.*, 1997; Saunders *et al.*, 1997; Cottingham *et al.*, 1999). *S. pombe* may not share this requirement for a destabilizing motor, or this function may be accomplished by other proteins expressed in the fission yeast cell. Third, both *klp5⁺* and *klp6⁺* are necessary for proper microtubule organization and cell morphology in several genetic backgrounds. As discussed below, this phenotype is particularly sensitive to the activity of cell cycle regulatory phosphatase *cdc25⁺*. However, no such functions have been reported for KIP3 in budding yeast. Finally, *klp5⁺* and *klp6⁺* are both essential for meiosis, as discussed below, whereas KIP3 is not (Cottingham and Hoyt, 1997; Miller *et al.*, 1998).

Microtubules and Cell Morphology

The establishment and maintenance of cell polarity and morphology is a microtubule-dependent process in fission yeast such that perturbations in microtubule organization can produce defects in cell shape and polarity (reviewed in Hagan, 1998; Chang, 2001). Previous work has demonstrated that shortening microtubule length, either by mutations in tubulin or tubulin-Cofactor genes (Umesono *et al.*,

1983; Grishchuk and McIntosh, 1999; Radcliffe *et al.*, 1999), by the addition of TBZ (Sawin and Nurse, 1998), or by deletion of microtubule-associated proteins (*mal3⁺*, Beinhauer *et al.*, 1997; *tea2⁺*, Browning *et al.*, 2000; *tip1⁺*, Brunner and Nurse, 2000; *dis1⁺* and *mtc1⁺*, Nakaseko *et al.*, 2001) leads to the formation of T-shaped cells. Results presented here suggest that an increase in microtubule length can cause bending at the growing ends of the cells, resulting in C- or J-shaped cells. This conclusion is supported by similar results reported for null mutants of *moe1⁺*, a component of the Ras1 signaling pathway (Chen *et al.*, 1999), and components of the γ -tubulin complex (Paluh *et al.*, 2000; Vardy and Toda, 2000). The changes in cell shape manifested in the *klpΔ* mutants described here occurred in genetic backgrounds and growth conditions that made the cells especially long, including several cell cycle arrest mutants and diploid strains. There may be a heightened sensitivity to changes in microtubule organization when the distance between the cell's ends is significantly increased. The T phenotype of *tea2Δ* cells is also enhanced when the cells are longer (Browning *et al.*, 2000).

The *klpΔ*-mediated changes in morphology are also enhanced by higher growth temperatures, as evidenced by the phenotype of *klpΔ* homozygous diploid cells grown at 36 versus 25°C. This result is consistent with the hypothesis that the phenotype arises as a result of hyperstabilized microtubules, because higher temperatures generally favor microtubule assembly. A similar temperature dependence has been reported for cell morphology defects observed in cells treated with TBZ (Sawin and Nurse, 1998). Because the cell cycle mutants used here to produce long cells are all temperature sensitive, it is not possible to distinguish clearly between the effects of temperature and cell length. Nonetheless, several observations argue that the effects we report are both temperature dependent and enhanced by cell length. Normal cell shapes are observed in both *klpΔ*, *cdc25-22* and *klpΔ* diploid cells at 25°C, despite the presence of abnormal microtubules, suggesting a temperature-dependent effect on cell morphology. On the other hand, the normal cell shape seen in *klpΔ* haploid cells at any temperature argues that increased cell length also contributes to the bent cell phenotype. Moreover, the bent-cell phenotype observed in all the cell cycle arrest mutants was greater as the cells continued to get longer in the arrest.

The formation of C-shaped cells in the *cdc25-22* background and of J-shaped cells in the *cdc10-V50* background is likely to be the consequence of monopolar (*cdc10-V50*) versus bipolar (*cdc25-22*) cell growth. The switch between these states, termed New-End Take Off, occurs at the beginning of G₂ (Mitchison and Nurse, 1985). Thus, the arrest points of *cdc10-V50* (G₁) and *cdc25-22* (late G₂) are on opposite sides of this event.

Examination of the microtubule cytoskeleton in the bent cells with *klp5Δ* and *klp6Δ* genotypes indicates that the interplay between cell elongation and the microtubule cytoskeleton is not a simple matter of microtubule geometry. First, the organization of the microtubules in the long *klpΔ*, *cdc25-22* and *klpΔ* diploid cells is distinct from either *klpΔ cdc10-V50* or *klpΔ cdc2-33* cells, and from previously published *ban* mutants (Verde *et al.*, 1995), although cell shape is similar in all of these cases. Second, the *cdc25-22* mutation had a profound effect on the organization of the microtu-

bules in the *klpΔ* background at permissive temperature, but cell morphology was not changed. Finally, the T morphology observed in *tea2Δ* cells is not rescued in *tea2Δ klpΔ* double and triple mutants, although the short-microtubule phenotype was at least partially rescued in these mutants (our unpublished data). The effect of *cdc25⁺* function on microtubule organization is discussed below, but the results discussed here indicate that certain kinds of changes in microtubules do not affect a cell's polar organization. Meanwhile, different changes in microtubule arrangement can produce the same net result on cell morphology.

cdc25⁺ and Microtubule Organization

Even a subtle disruption in the function of *cdc25⁺* leads to a profound rearrangement of the microtubule cytoskeleton in the absence of either *klp5⁺* or *klp6⁺* (Figure 5). Cells carrying the temperature-sensitive allele *cdc25-22* together with the *klpΔ* mutations had extended microtubule arrays that often curled around the ends of the cells, even at permissive temperature. Several lines of evidence indicate that this effect is not simply the result of the increased length of the *cdc25-22* cells. First, the effect was not observed in either *cdc2-33* or *cdc10-V50* cells, although they are similar in size to *cdc25-22*. Second, curled microtubules were also observed in *klpΔ, wee1-50* cells, which are shorter than wild-type cells. Third, the microtubules are also not likely to be curling simply due to a physical barrier presented by the ends of the cells, because the microtubules often curled before they reached the ends of the cells. Moreover, the shorter *klpΔ, wee1-50* cells have a less severe phenotype than that observed in *cdc25-22* cells, contrary to what might be expected if hyperstable microtubules were confined to a smaller space. These results favor a regulatory interaction between the microtubules and *cdc25⁺* that is normally masked by the presence of *klp5⁺* and *klp6⁺*. The interaction between *klpΔ* and both *wee1-50* and *cdc25-22* may seem enigmatic because these mutants have opposite effects on cell size and are antagonistic to each other in their regulatory pathway (reviewed in Forsburg and Nurse, 1991). It is possible, however, that any alteration in the balance between Cdc25p and Wee1p activity leads to aberrant microtubules.

It is also possible that the phenotype observed in *klpΔ, cdc25-22* mutants is dependent on cells being in G₂, and not on *cdc25⁺* activity, alone. This could explain why the *klpΔ, cdc10-V50* mutants do not have the same phenotype. However, this seems unlikely because the majority of the *cdc2-33* cells are also arrested in G₂, and their phenotype more closely resembled that of *cdc10-V50*. Furthermore, the *klpΔ, wee1-50* cells have a shortened G₂ stage, and yet they also have the bent microtubule phenotype. At present it is impossible to rule out other G₂-specific factors because the substrate for either *cdc25⁺* or *wee1⁺* that affects microtubule behavior remains unknown. Because the phenotype is present in null alleles of *klp5⁺* and *klp6⁺*, it is likely that the potential substrates revealed here include proteins other than these two kinesins. The absence of the curled microtubules in the *klpΔ, cdc2-33* mutants suggests that the mechanism is independent of MPF activity. Rather, it seems likely that *cdc25⁺* is interacting with an unidentified gene whose activity is normally buffered by *klp5⁺* or *klp6⁺*. A role for *cdc25⁺* in regulating microtubule behavior is also indicated by interactions between the *cdc25-22* allele and several other

kinesins, including *klp2⁺* (Sweezy and McIntosh, personal communication), and *tea2⁺* (Browning *et al.*, 2000). Tubulin metabolism involves many different classes of genes, so the candidates for such a locus are numerous.

Meiosis

Our data indicate that both Klp5p and Klp6p play a major role in the production of viable spores from meiosis. Crosses among *klp5⁺* and *klp6⁺* null mutants produced zygotically with abnormal morphologies and extremely low spore viability. Azygotically induced from stable diploid cells displayed a similar loss in spore viability. The similarity in phenotypes observed between zygotically and azygotically spore formation suggests that these mutations lead to meiotic defects downstream from conjugation and karyogamy. The precise point(s) of meiotic failure remains to be determined. The intermediate phenotype in heterozygous crosses between *klp5Δ* and *klp6Δ*, versus heterozygous crosses between either *klp5Δ* or *klp6Δ* and wild type, does suggest an unusual, interallelic form of haplo-insufficiency for these two kinesins in meiosis.

The essential role for both *klp5⁺* and *klp6⁺* in the meiotic life cycle contrasts strongly with the behaviors found for vegetative growth. *klp5⁺* and *klp6⁺* are the fifth and sixth motor proteins demonstrated to have a major role in fission yeast meiosis (*cut7⁺*, Hagan and Yanagida, 1990; *dhc1⁺*, Yamamoto *et al.*, 1999; *pk11⁺* and *klp2⁺*, Troxell *et al.*, 2001), whereas only *cut7⁺* is essential for mitosis (Hagan and Yanagida, 1990). This indicates that fission yeast meiosis is very sensitive to the normal functioning of the microtubule cytoskeleton compared with vegetative growth. It remains to be determined whether the activity of each kinesin, per se, is distinct in these two life cycle stages, or whether the differences between meiosis and mitosis arise from differences in the mechanical requirements or functional redundancies among the motor proteins used to achieve these mechanics, or even from the checkpoints that operate in each life cycle stage.

ACKNOWLEDGMENTS

We thank Drs. Heidi Browning and Katya Grishchuk for helpful suggestions and critical reading of the manuscript. We thank Da Qiao Ding for the pDQ105 (GFP- α -tubulin) plasmid. We thank Christy Fillman for help constructing the kinesin quadruple mutants. This work was supported by National Institutes of Health grant GM-33787 to J.R.M., who is a Research Professor of the American Cancer Society.

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