The Kinesin-related Proteins, Kip2p and Kip3p, Function Differently in Nuclear Migration in Yeast

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The roles of two kinesin-related proteins, Kip2p and Kip3p, in microtubule function and nuclear migration were investigated. Deletion of either gene resulted in nuclear migration defects similar to those described for dynein and *kar9* mutants. By indirect immunofluorescence, the cytoplasmic microtubules in *kip2* were consistently short or absent throughout the cell cycle. In contrast, in *kip3* strains, the cytoplasmic microtubules were significantly longer than wild type at telophase. Furthermore, in the *kip3* cells with nuclear positioning defects, the cytoplasmic microtubules were misoriented and failed to extend into the bud. Localization studies found Kip2p exclusively on cytoplasmic microtubules throughout the cell cycle, whereas GFP-Kip3p localized to both spindle and cytoplasmic microtubules. Genetic analysis demonstrated that the *kip2 kar9* double mutants were synthetically lethal, whereas *kip3 kar9* double mutants were viable. Conversely, *kip3 dhc1* double mutants were synthetically lethal, whereas *kip2 dhc1* double mutants were viable. We suggest that the kinesin-related proteins, Kip2p and Kip3p, function in nuclear migration and that they do so by different mechanisms. We propose that Kip2p stabilizes microtubules and is required as part of the dynein-mediated pathway in nuclear migration. Furthermore, we propose that Kip3p functions, in part, by depolymerizing microtubules and is required for the Kar9p-dependent orientation of the cytoplasmic microtubules.

INTRODUCTION

The migration and orientation of the nucleus and spindle are critical events in mitosis in eukaryotic cells. In many organisms the position of the spindle determines the position of the cleavage furrow at cytokinesis. For example, in *Caenorhabditis elegans*, the reorientation of the spindle in response to a cortical signal is essential for establishing the orientation of the second cell division (Hyman, 1989). In the budding yeast, *Saccharomyces cerevisiae*, cytokinesis is not determined by the orientation of the spindle. Instead, the spindle must become oriented with respect to the growth axis

of the cell to allow elongation of the spindle into the growing bud.

In *S. cerevisiae*, the nucleus does not break down during cell division and the microtubule organizing centers (or spindle pole bodies, SPBs) are embedded in the nuclear envelope. Spindle microtubules extend from the SPB into the nucleus, and cytoplasmic microtubules extend outward toward the cell cortex (Byers and Goetsch, 1975). Early in G1/S the nucleus rotates to orient the SPB toward the bud (Snyder *et al.*, 1991). The SPB then duplicates to initiate formation of the bipolar spindle. By the end of G₂, before the onset of anaphase, the nucleus migrates to the neck of the mother–bud junction. At this point, one SPB is oriented toward the bud neck with the cytoplasmic microtubules extending into the bud (Byers and Goetsch, 1975). With the onset of anaphase the nucleus both elongates and translocates through the bud neck (Kahana *et al.*, 1995; Yeh *et al.*, 1995). After the separation

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of the nuclear material at late anaphase and telophase, cytokinesis occurs.

Failure in nuclear orientation and migration can cause severe defects in the partitioning of the nuclear material during the subsequent mitosis. Both the cytoplasmic microtubules and the actin cytoskeleton are required for proper nuclear positioning. Disruption of either results in aberrant spindle orientation before anaphase and a failure of nuclei to migrate into the bud neck. Initially, such defects result in the accumulation of binucleate mother cells (Jacobs *et al.*, 1988; Palmer *et al.*, 1992; Sullivan and Huffaker, 1992). The complete absence of cytoplasmic microtubules leads to the formation of multinucleate mother cells with multiple anucleate buds (Sullivan and Huffaker, 1992).

Several microtubule-associated proteins have been shown to be required for efficient nuclear migration. For example, the minus end-directed microtubule motor protein dynein is thought to act by generating the forces required for translocation of the nucleus (Eshel *et al.*, 1993; Li *et al.*, 1993). Deletion of the dynein gene (*DHC1/DYN1*) causes an abnormal placement of the spindle relative to the bud neck, resulting in the occurrence of anaphase entirely within the mother cell. Consequently, as many as 10–15% of the mother cells in the *dhc1*Δ deletion strain are binucleate. One model of nuclear migration places dynein at a cortical site such that its minus-end motor activity would pull on the cytoplasmic microtubules to “tow” the nucleus through the bud neck (Eshel *et al.*, 1993; Li *et al.*, 1993). Consistent with this hypothesis, observations in live cells demonstrated that the cytoplasmic microtubules spend a significant period of time in contact with the bud cortex, and the cortical association is coordinated with the budward movement of the spindle (Carminati and Stearns, 1997). Furthermore, in dynein-deficient cells, microtubule-associated movement and microtubule dynamics were significantly altered (Carminati and Stearns, 1997). However, recent experiments with dynein-green fluorescent protein (GFP)¹ hybrids suggest that dynein may be localized along the length of the cytoplasmic microtubules (Shaw *et al.*, 1997). If so, the dynein-dependent force may result from interactions with cytoskeletal elements other than the cortex and act along the length of the microtubules.

Two genes, *ACT5* and *JNM1*, are thought to act with dynein as part of the dynactin complex. *ACT5* is the yeast homologue of *ARP1* that encodes a component of the vertebrate dynactin complex (Schafer *et al.*, 1994). *JNM1* has been proposed to correspond to the vertebrate p50 dynactin component (Geiser *et al.*, 1997). Mutations in either gene result in spindle orientation and nuclear migration defects similar to those

observed in dynein heavy chain mutants (Eshel *et al.*, 1993; Clark and Meyer, 1994; McMillan and Tatchell, 1994; Muhua *et al.*, 1994). Double mutants with either of these genes and a dynein deletion are no worse than single mutants alone, indicating that *ACT5* and *JNM1* function in the same pathway as dynein for nuclear migration (Geiser *et al.*, 1997; Muhua *et al.*, 1994; Tatchell, personal communication).

The cortical protein Kar9p functions in nuclear migration in a pathway separate from, yet partially redundant with, dynein. The *kar9*Δ mutants exhibit mitotic defects similar to dynein and are synthetically lethal in combination with *dhc1*, *jnm1*, and *act5* deletion mutants (Miller and Rose, 1998). Unlike the *dhc1* mutant, *kar9* mutants have misoriented cytoplasmic microtubules in both mitosis and mating. In both cases it is likely that microtubule misorientation results in disrupted nuclear positioning (Miller and Rose, 1998). GFP-tagged Kar9p localizes to a single cortical spot at the tip of the bud and at the tip of mating projections. Because GFP-Kar9p localization at the cell cortex is independent of microtubules, yet required for their orientation, Kar9p may function as a cortical target for the capture of the cytoplasmic microtubules. Capture and stabilization of the cytoplasmic microtubules would then provide a mechanism for the orientation of the microtubules and the mitotic spindle (Miller and Rose, 1998).

It was initially surprising that dynein's role in positioning the nucleus during nuclear migration is not essential for life. Several general models can be advanced to explain this observation. First, random motion of the nucleus, together with spindle elongation, would allow the nucleus to enter the bud during anaphase. While the proper orientation might occur infrequently, elongation into the bud would be irreversible. Second, other microtubule-dependent motors might provide the force for nuclear movement. Third, the intrinsic dynamic properties of microtubules might provide sufficient force for movement. Support for the latter two hypotheses comes from the observation that the *tub2-401* mutation selectively destabilizes the cytoplasmic microtubules and leads to a much more severe nuclear migration defect than the dynein deletion. Candidate motor proteins that might provide compensatory or redundant forces in the absence of dynein include the kinesin-related motor proteins. Of the six kinesin-related genes in *S. cerevisiae*, three (*KAR3*, *KIP1* and *CIN8*) have been shown previously to play a role in spindle function (Meluh and Rose, 1990; Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders *et al.*, 1997).

The kinesin-related gene *KIP2* was previously identified in a screen for kinesin-related genes using degenerate PCR primers to conserved regions of kinesin-related motor domains (Roof *et al.*, 1991, 1992). Unlike conventional kinesin, the Kip2p motor domain is positioned in the center of the molecule. Preliminary

¹ GFP, green fluorescent protein; HA, hemagglutinin; SBP, spindle pole body;

work reported that *kip2* mutants exhibited a defect in nuclear migration and were not synthetically lethal with dynein mutants (Miller and Rose, 1995). Subsequent work confirmed the role of Kip2p in nuclear migration (Cottingham and Hoyt, 1997). The kinesin-related gene *KIP3* was identified during the completion of the yeast genome-sequencing project and encodes an 805-amino acid protein with an N-terminal motor domain. This and concurrent work (Cottingham and Hoyt 1997; DeZwaan *et al.*, 1997) demonstrate that *kip3Δ* mutants are also defective for nuclear migration during mitosis. DeZwaan *et al.* (1997) showed that nuclear positioning is random and that the mitotic spindle is misoriented in preanaphase *kip3Δ* cells. Based upon genetic and morphological data, DeZwaan *et al.* (1997) proposed that Kip3p and dynein act at different temporal steps to complete anaphase. In addition, Cottingham and Hoyt (1997) provided genetic evidence that suggests that Kip2p and Kip3p act antagonistically to position the mitotic spindle.

The genetic and morphological studies presented in this paper confirm that both Kip2p and Kip3p affect nuclear migration and revealed that they do so via different mechanisms. Our conclusions are based primarily upon the differences observed for *kip2Δ* and *kip3Δ* with respect to genetic interaction profiles, microtubule morphology, and cellular localization. The *kip3Δ* mutation was synthetically lethal with a deletion mutation in dynein (also shown by Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997) and with deletions in components of the dynactin complex. In contrast, *kip2Δ* mutations were not synthetically lethal in combination with deletions in dynein (also shown by Cottingham and Hoyt, 1997) or with deletions in components of the dynactin complex. Conversely, the *kip2Δ* mutations were found to be synthetically lethal with *kar9Δ*, whereas *kip3Δ kar9Δ* double mutants were viable. Furthermore, Kip2p was localized to the cytoplasmic microtubules throughout the cell cycle. In contrast, Kip3p was found on both the spindle and cytoplasmic microtubules (also shown by DeZwaan *et al.*, 1997). These results support the view that there are at least two partially redundant pathways for nuclear migration, one of which requires Kar9p and the second of which requires dynein. Overall, our findings add to our expanding knowledge of the components involved in positioning the nucleus during the cell cycle.

MATERIALS AND METHODS

Yeast Strains, Microbial Techniques, and Growth Assays

Yeast strains used in this study are listed in Table 1. Plasmids and bacterial strains are listed in Table 2. Yeast media and standard genetic techniques were essentially as previously described (Rose *et al.*, 1990).

Double-deletion mutants were created by standard genetic techniques. The growth phenotypes of spores were scored on the second and third day of growth after tetrad dissection. Colonies were classified as showing normal growth, microcolonies, or no growth. To examine the temperature sensitivity of the germinating spores, 10 tetrads from each cross were dissected onto YPD plates and incubated at 37°, 35°, 23°, 16°, and 14°C.

The growth rates of the *kip3Δ::HIS3* strain (MS4516) and the wild-type strain (MS1554) were evaluated as follows. The strains were grown in YPD medium at 30°C until the cultures were in the exponential phase of growth. The cultures were then diluted 10-fold into YPD that had been preincubated at 37°, 30°, 23°, or 14°C. Optical density readings were taken at various time points to estimate cell numbers. Tetrads from a heterozygous *kip3Δ/KIP3* diploid were dissected on YPD medium, and identical plates containing 10 tetrads each were incubated at 14°, 16°, 23°, 30°, and 37°C. At all temperatures the *kip3Δ* strain grew identically to wild type.

DNA Manipulations

For PCR analysis, genomic DNA was prepared from strains MS4516 and MS1554 by a modification of the CsCl method (Rose *et al.*, 1990). DNA preparations for transformations and plasmid construction were made as described previously (Rose *et al.*, 1990). Plasmid transformations into yeast were carried out by the lithium acetate method (Ito *et al.*, 1983). Transformations of bacteria were performed by standard electroporation techniques. Gene disruptions were done as described previously (Rothstein, 1991).

Strain Construction

KIP2 was previously identified using a PCR-based homology screen (Roof *et al.*, 1992). *KIP3* was identified by the yeast genome-sequencing project and corresponds to ORF YGL216W. A strain lacking the entire *KIP3*-coding region was created by the one-step gene replacement method (Rothstein, 1991). The disruption fragment was generated by PCR using the following two oligonucleotides (Syn/Seq Facility, Princeton University, Princeton, NJ). The primer sequences were (*KIP3* sequence is in italics, *HIS3* is in roman font): 5'-TAC TTG AGT TTT CTT TCC AGC TGT ATA CTA TTG ACA CTA ACA TGC CGT TTT AAG AGC TTG GTG-3'; and 5'-GAA AGA AGT TAT ATT CGA TAG TTT ACG TAG GAT ATG TAT GGT CGA GTT CAA GAG AAA AAA-3'. Plasmid pRS403 (Sikorski and Hieter, 1989) was used as the template for the *HIS3* portion of the PCR construct. Transformation of a haploid strain (MS1554) produced a *kip3Δ::HIS3* strain (MS4516). The replacement of the entire *KIP3* coding region from the ATG start site through the last codon of *KIP3* by *HIS3* was confirmed at both ends by PCR analysis. All *kip3Δ* strains described in this study were derived from backcrosses of MS4516.

MS4262, a *dhc1Δ::URA3* disruption in a S288C isogenic strain was made as described elsewhere (Miller and Rose, 1998) using pBR2-1U (Li *et al.*, 1993). MS4262 was backcrossed once to create MS4304, a strain with the required auxotrophic markers. MS4321, the *jnm1Δ::LEU2* disruption in a S288C isogenic strain, was made as described elsewhere (Miller and Rose, 1998) using pJM1432 (McMillan and Tatchell, 1994). MS4321 was backcrossed once to obtain strain MS4692 used in the analysis. An *act5Δ1-366::TRP1* disruption strain BY397 (from Sean Clark, Princeton University) was generated from MS17 using the plasmid pTRP17-20 (Clark and Meyer, 1994) digested with *SacI* and *XhoI*. This strain was backcrossed once to obtain MS4701. MS4734, a *bik1Δ* strain, was made as described previously (Miller and Rose, 1998) using plasmid pVB17 (Fink Laboratory, Whitehead Institute, Cambridge, MA). This strain was backcrossed once to obtain MS4915.

Nuclear and Microtubule Morphology in Mitotic and Mating Cells

To assay for defects in mitosis, strains were grown to exponential phase at 30°, 14°, and 12°C. After 4 h at 30°C (24 h at 14° and 12°C),

cells were centrifuged and fixed in methanol:acetic acid (3:1) for 30 min on ice. After washing in PBS, cells were stained with the fluorescent DNA dye DAPI at 1 $\mu\text{g}/\text{ml}$ for 30 min. The cells were then analyzed microscopically using differential interference contrast optics to assess the cellular morphology and UV fluorescence to evaluate the nuclear morphology.

To assay karyogamy, selected strains of opposite mating types were grown to early exponential phase at 23°C, mixed in a 1:1 ratio, and collected by filtration on 0.45- μm filters (Millipore, Bedford, MA). The mating mixtures on the filters were incubated on YPD media for 2.5 h at 30°C. Cells were then resuspended in PBS, fixed in methanol-acetic acid, and stained with DAPI to visualize nuclear material as described above.

To visualize microtubules, indirect immunofluorescence was carried out as described previously (Rose *et al.*, 1990). Selected strains were grown to exponential phase at 30°C and shifted to 14°C for 16–24 h. Microtubules were visualized with the rat anti-tubulin antibody YOL1/34 (Accurate Chemical and Scientific, Westbury, NY) undiluted, and FITC-conjugated goat anti-rat secondary antibody (Amersham, Arlington Heights, IL) at a 1:100 dilution.

Localization of Kip2p

To localize Kip2, in some experiments a hemagglutinin (HA) epitope-tagged form of KIP2 was used. A fragment coding for a triple HA tag with *Hind*III ends was synthesized by PCR using the GTEPI plasmid (from Bruce Futcher, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), as template. The following primers were used: 5'-GCC CAA GCT TAT ACC CAT ACG AT-3', and 5'-GCC TTA AGC TTG CAG CGT AAT CTG G-3'. The KIP2-containing CEN plasmid pMR3144 was cut with *Hind*III, and the HA-containing insert was ligated into it to produce the plasmid pMR3777. To create a 2 μ version of this construct, pMR3777 was cut with *Kpn*I and *Sac*I, and the 2.6-kilobase (kb) fragment containing KIP2::HA was gel purified and ligated to pRS426 (Sikorski and Hieter, 1989), to produce pMR3779. To construct a nonpeptide-tagged control plasmid, the *Kpn*I/*Sac*I fragment containing KIP2 was excised from pMR3144 and ligated to pRS426 (Sikorski and Hieter, 1989), creating the 2 μ KIP2 URA3 plasmid, pMR3778.

To localize Kip2p-HA, a *kip2 Δ* strain (MS2354) containing pMR3779 was fixed using a modified procedure described elsewhere (Roberts *et al.*, 1991). Five-milliliter cultures of cells grown to midexponential phase were fixed in 4% paraformaldehyde for 4 h at 23°C. No preceding formaldehyde fixation was used. Cells were then incubated in TEB (200 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% 2-mercaptoethanol, prepared fresh) for 10 min at 23°C. Cells were resuspended in SPM (1.2 M sorbitol, 50 mM potassium phosphate, pH 7.3, 1 mM MgCl₂) and stored at 4°C for 16 h. The cell walls were digested for 1 h at 30°C with 50 μl Glusulase (DuPont, Wilmington, DE) and 15 μl Zymolyase 100T (10 mg/ml) (ICN Immunobiologicals, Lisle, IL). Cells were extracted with 1% SDS/1.2 M sorbitol/PBS for 10 min at 23°C. The extracted cells were then diluted fivefold with 1.2 M sorbitol/PBS and washed three times with 1.2 M sorbitol/PBS. The cells were then attached to poly-L-lysine coated coverslips and blocked with BSA/PBS (5 mg/ml) for 10 min. Monoclonal HA antibody (12CA5) was used at 1:300 dilution and goat anti-mouse FITC-conjugated secondary was used at 1:25 dilution. Both antibodies were preabsorbed using a *kip2 Δ* strain.

In other experiments a GFP-Kip2p hybrid was used to localize Kip2p. For this purpose, plasmid pMR3769 (M. Elowitz and S. Leibler, Princeton University) containing *Aquoreus victoria* GFP mutant 2 (Cormack, 1996) was used as template in a PCR reaction to generate a GFP fragment flanked by *Hind*III recognition sites. The following oligonucleotides were used as primers: 5'-CGG CGC CCA AGC TTG ATG AGT AAA GGA GAA G-3', and 5'-CCC AAG CTT TTG TAT AGT TCA TCC ATG-3'. The resulting PCR product was digested with *Hind*III and ligated into the *Hind*III site of KIP2 on pMR3144, creating pMR3889. This construct complemented the growth defect of a *kip2 Δ kar9 Δ* double mutant, produced by trans-

forming and sporulating the *kip2 Δ /KIP2 kar9 Δ /KAR9* heterozygous diploid, MS5210.

To visualize GFP-Kip2p, strains containing pMR3889 were grown at 23°C to early exponential phase in synthetic complete media minus uracil. To assess nuclear material, cells were fixed in 3.7% formaldehyde for 5 min, washed twice in PBS, stained with DAPI for 5 min, and washed in PBS twice as was described above. Cells were viewed using a Zeiss Axiophot microscope equipped with a High Q FITC filter set (no. 41001 from Chroma Technology, Brattleboro, VT), a 100 $\times$ plan-neufluor objective (1.3 N.A.) (Carl Zeiss, Thornwood, NY), and a cooled charge-couple device camera (Princeton Instruments, Princeton, NJ) connected to a Hamamatsu Video Camera 3200 and a Hamamatsu Image Processor C2400 (Hamamatsu, Hamamatsu City, Japan).

Visualization of GFP-Kip3p and GFP-Kar9p

The KIP3 gene was amplified with PCR using the following flanking primers: 5'-CCG CCG TCG ACT ATT GAC ACT AAC ATG-3' and 5'-CGG GAT CCG CTG GCG GAA AGA AGT TA-3'. The PCR product was digested with *Sal*I and *Bam*HI and ligated into a *P_{GAL}-GFP#1* vector pB1893 (Corey Davis and James Broach, Princeton University) cut with the same enzymes to create the *P_{GAL}-GFP-KIP3* construct, pMR3635.

A wild-type strain (MS1554) containing the *P_{GAL}-GFP-KIP3* construct was grown at 30°C to early exponential phase in synthetic complete medium minus leucine containing 2% raffinose. GFP-Kip3p expression was induced by the addition of 2% galactose for 3–4 h. The localization was determined using the microscopy conditions described for GFP-Kip2p.

The localization of GFP-Kar9p was performed and scored as described previously (Miller and Rose, 1998) except that glucose-modulated galactose inductions for Kar9p-GFP expression were not used; instead, the expression was fully induced using 2% galactose for 2–3 h.

RESULTS

kip2 Δ and kip3 Δ Mutants Display Defects in Mitosis but Not in Karyogamy

To assess the functions of the two kinesin-related genes, KIP2 and KIP3, strains containing deletions of each were constructed. In both cases, the deletion strains were viable and exhibited wild-type growth rates at a wide range of temperatures. We next examined them for more subtle defects in cell cycle progression. During mitosis in wild-type cells, the nucleus migrates to the neck of the mother–bud junction and anaphase occurs through the space of the neck (Kahana *et al.*, 1995; Yeh *et al.*, 1995). In both the *kip2 Δ* and *kip3 Δ* strains, a variety of nuclear migration defects could be observed. These included 1) large-budded cells with a single nucleus that had failed to migrate to the bud neck; 2) large-budded cells with anaphase occurring exclusively within the mother cell; and 3) large-budded cells with the mother cell containing two nuclei (Figure 1 and Table 3A). Such aberrant cells occurred infrequently in the isogenic wild-type strain (~2%). In contrast, at 30°C, ~7% of the *kip2 Δ* cells exhibited nuclear migration defects (Table 3A). At 12°C, the frequency of aberrant cells increased to ~20%, while in the wild-type strain the frequency remained at 2%. Most of the increase occurred in the

Table 1. Yeast strains used in this study

Strain	Genotype	Source
MS17	<i>MATα trp1-Δ1 ura3-52 ade2-101</i>	
MS52	<i>MATα leu2-3 leu2-112 trp1-Δ1 ura3-52</i>	
MS660	<i>MATα kar3-101::LEU2 leu2-3 leu2-112 ura3-52 his4-539 trp1-Δ1</i>	
MS1554	<i>MATa ura3-52 leu2-3 leu2-112 ade2-101 his3-Δ200</i>	
MS2305	<i>MATα kip1-Δ::URA3 ura3-52 leu2-3 leu2-112 trp1-Δ1</i>	
MS2309	<i>MATa kip2-Δ1::URA3 ura3-52 leu2-3 leu2-112 ade2-101 his3-Δ200</i>	
MS2310	<i>MATα kip2-Δ1::URA3 ura3-52 leu2-3 leu2-112 trp1-Δ1</i>	
MS2354	<i>MATα kip2-Δ::TRP1 ura3-52 leu2-3 leu2-112 trp1-Δ1</i>	
MS2442	<i>MATα kip2::TRP1 trp1-Δ1 leu2-3 leu2-112 his3-Δ200 ura3-52 ade2-101</i>	
MS4062	<i>MATα kar9-Δ1::LEU2 leu2-3 leu2-112 trp1-Δ1 ura3-52</i>	
MS4262	<i>MATα dhc1-Δ::URA3 leu2-3 leu2-112 trp1-Δ1 ura3-52</i>	
MS4263	<i>MATa kar9-Δ1::LEU2 leu2-3 leu2-112 ade2-101 his3-Δ200 ura3-52</i>	
MS4304	<i>MATa dhc1::URA3 ura3-52 leu2-3 leu2-112 trp1-Δ1 his3-Δ200</i>	
MS4308	<i>MATa kip2-Δ::URA3 trp1-Δ1 ade2-101 his3-Δ200 ura3-52 leu2-3 leu2-113</i>	
MS4316	<i>MATα kar9-Δ1::LEU2 leu2-3 leu2-112 ura3-52 ade2-101 his3-Δ200</i>	
MS4321	<i>MATa jnm1-Δ::LEU2 ura3-52 leu2-3 leu2-112 ade2-101 his3-Δ200</i>	
MS4516	<i>MATa kip3-Δ::HIS3 his3-Δ200 ade2-101 leu2-3 leu2-112 ura3-52</i>	
MS4586	<i>MATα act5-Δ::HIS3 trp1-Δ63 lys2-801 his3-Δ200 ade2-101 ura3-52 leu2</i>	
MS4600	<i>MATα dhc1-Δ::URA3 ura3-52 ade2-101 trp1-Δ1 his4-539</i>	
MS4648	<i>MATα kip3-Δ::HIS3 kar9-Δ::LEU2 his3-Δ200 ade2-101 leu2-3 leu2-112 ura3-52</i>	
MS4654	<i>MATα kip3-Δ::HIS3 kar9-Δ::LEU2 his3-Δ200 ade2-101 leu2-3 leu2-112 ura3-52</i>	
MS4691	<i>MATa kip3-Δ::HIS3 ura3-52 his3-Δ200 leu2-3 leu2-112 trp1</i>	
MS4692	<i>MATa jnm1-Δ::LEU2 leu2-3 leu2-112 his3-Δ200 ura3-52 trp1-Δ1</i>	
MS4701	<i>MATα act5-Δ::TRP1 trp1-Δ1 his3-Δ200 ade2-101 ura3-52</i>	
MS4719	<i>MATa/MATα kip3-Δ::HIS3/kip3-Δ::HIS his3-Δ200/his3-Δ200 ade2-101/ADE2 leu2-3/leu2-3 leu2-112/leu2-112 TRP1/trp1-Δ1 ura3-52/ura3-52</i>	
MS4720	<i>MATa/MATα ura3-52/ura3-52 leu2-3/leu2-3 leu2-112/leu2-112 ade2-101/ADE2 his3-Δ200/HIS3 trp1-Δ1/TRP1</i>	
MS4734	<i>MATα bik1-Δ::TRP1 leu2-3 leu2-113 trp1-Δ1 ura3-52</i>	
MS4903	<i>MATa dhc1::LEU2 ura3-52 leu2-3 leu2-112 ade2-101 his3-Δ200</i>	
MS4915	<i>MATα bik1::TRP1 leu2-3 leu2-112 trp1-Δ1 ura3-52 his3-Δ200 ade2-101</i>	
MS5001	<i>MATa dhc1-Δ::URA3 ura3-52 leu2-3 leu2-112 ade2-101 his3-Δ200</i>	
MS5210	<i>MATa/MATα kar9-Δ1::HIS3/KAR9 kip2-Δ::TRP1/KIP2 leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52 ade2-101/ADE2 his3-Δ200/his3-Δ200 trp1-1/trp1-Δ1</i>	
MS5214	<i>MATa kip2-Δ::TRP1 dhc1-Δ::URA3 ura3-52 leu2-3 leu2-112 trp1-Δ1 his3-Δ200</i>	
BY397	<i>MATα act5-Δ1-366 trp1-Δ1 ade2-101 ura3-52</i>	Clark
APY4ΔD5	<i>MATa smy1Δ::URA3 ura3-52 his6 leu2-3 leu2-112 trp1 ade2</i>	Brown
MAY2058	<i>MATα cin8Δ::LEU2 leu2-3 leu2-112 his3-Δ200 ura3-52 lys2-801</i>	Hoyt
MAY2059	<i>MATa cin8Δ::LEU2 his3-Δ200 leu2-3 leu2-112 ura3-52 ade2-101</i>	Hoyt

Unless indicated otherwise, all strains are from this laboratory.

class of large-budded cells with two nuclei in the mother cell (e.g., for the *kip2Δ::TRP1* strain, the number of binucleate mother cells increased from 2 to 16%).

For the *kip3Δ* strain at 30°C, 9% of the culture (25% of the large-budded cells) displayed defects in nuclear positioning (Table 3B) compared with 3% for wild type. At 14°C, the frequency of aberrant cells increased to 15% (27% of large-budded cells). While the total increase in the frequency of aberrant cells was not as large as the *kip2Δ* strain, the specific spectrum of defects for the *kip3Δ* strain shifted dramatically. At 30°C, in most of the aberrant cells the nuclei failed to migrate to the bud neck. In contrast, at 14°C most of the aberrant cells were binucleate mother cells (increased from 0 to 18%). In addition, the percentage of cells that were unbudded binucleates increased from 0 to 5% at 14°C (our unpublished observations). Growth in the

cold also caused a shift in the cell cycle distribution as indicated by bud size. At 30°C, 33% of *kip3Δ* mutants were large budded; at 14°C, 52% were large budded (Table 3B). Despite these mitotic defects, the *kip3Δ* mutants did not show any growth defects at a variety of temperatures compared with wild type (see MATERIALS AND METHODS).

Clearly, both the *kip2* and *kip3* mutant strains displayed nuclear positioning defects in mitosis. For this reason, we wanted to determine whether these genes also played a role in karyogamy, another cellular function involving nuclear migration. During mating, cells arrest in G₁ in response to mating pheromones and initiate polarized growth toward each other. The intervening cell wall breaks down and the two cells fuse. The zygotic nuclei then migrate toward each other and fuse, forming a diploid zygote. This movement is microtubule dependent and requires the Kar3p motor

protein (Meluh and Rose, 1990; Kurihara *et al.*, 1994; Marsh and Rose, 1997). As a direct assay for nuclear fusion, microscopic examination of zygotes was performed. In wild-type zygotes, more than 98% have a single fused nucleus before the appearance of the first bud. In matings of *kip3Δ* crossed to wild type and matings of *MATα kip3Δ* crossed to *MATα kip3Δ*, greater than 95% of the unbudded zygotes contained a single fused nucleus. Similar microscopic analyses of *kip2Δ* matings revealed no defect in nuclear fusion, confirming previously reported results using a quantitative mating assay (Roof *et al.*, 1992). Thus, *kip2Δ* and *kip3Δ* mutants do not have karyogamy defects. These results confirm that Kar3p is the sole kinesin responsible for nuclear migration during mating (Meluh and Rose, 1990).

Distinct Microtubule Morphologies in *kip2Δ* and *kip3Δ*

Since nuclear migration and spindle orientation have been shown to depend on the cytoplasmic microtubules (Sullivan and Huffaker, 1992), we next examined whether deletions in either *KIP2* or *KIP3* caused altered microtubule morphology. In wild-type cells at telophase, the mitotic spindle aligns along the mother-bud axis, extending directly from the nuclear material in the mother cell through the nuclear material in the bud (Figure 2A). The cytoplasmic microtubules at this stage appeared as short extensions at one or both ends of the spindle in 79% of the wild-type cells (Figure 2A and Table 4A). One striking feature of the *kip2Δ* cultures grown at 14°C was the absence of the cytoplasmic microtubules in 53% of the telophase cells (Figure 2D and Table 4A). In contrast, in wild-type cultures only 14% of the telophase cells displayed no discern-

ible cytoplasmic microtubules. These results suggest that Kip2p may play a role in stabilizing the cytoplasmic microtubules at this stage of the cell cycle. Interestingly, in the *dhc1Δ* strain, 22% of the cells had no obvious cytoplasmic microtubules, indicating that dynein may also play a minor role in stabilizing the cytoplasmic microtubules. In contrast to *kip2Δ* and *dhc1Δ*, in the *kip3Δ* culture only 5% of the telophase cells lacked cytoplasmic microtubules (Figure 2, M–U, and Table 4A). Indeed, under these conditions 35% of the *kip3Δ* cells had abnormally long cytoplasmic microtubules (Figure 2 M and Table 4A). This aberrant morphology was not observed in the *kip2Δ* and *dhc1Δ* mutants or the wild-type strain. The long microtubules most often extended from the bud tip back into the mother cell (Figure 2M). Regardless of their abnormal microtubule phenotypes, more than 80% of the *kip3Δ* cells had segregated their nuclei normally. The effect on microtubules was temperature dependent because the *kip3Δ* cultures grown at 30°C appeared to have normal microtubules.

In addition to the cells showing a normal telophase, we specifically examined the microtubules in cells showing abnormal nuclear morphologies (Table 4, B and C, and Figure 2, G, J, P, and S). In the wild-type strain at 14°C, 28% of the cells in which mitosis was occurring within the mother cell had no detectable cytoplasmic microtubules (Table 4B). However, in the *kip2Δ* strain, 70–82% of the cells had no detectable cytoplasmic microtubules, regardless of whether nuclear division had been completed (Figure 2, G and J, and Table 4, B and C).

The morphology of the cytoplasmic microtubules in the *kip3Δ* mutants was strikingly different from that of the *kip2Δ* mutants. In anaphase, the percent of *kip3Δ* cells without cytoplasmic microtubules was virtually identical to wild type. However, many of the *kip3Δ* mutants had cytoplasmic microtubules that were misoriented and failed to extend into the bud (Figure 2, P–U, and Table 4, B and C). In the *kip3Δ* cells in which anaphase was occurring within the mother cell, 24% exhibited misoriented cytoplasmic microtubules. This frequency was similar to the 30% misoriented cytoplasmic microtubules found in the *kar9Δ* mutant (Miller and Rose, 1998). When two nuclei were present within the mother cell, 72% of *kip3Δ* cells had misoriented cytoplasmic microtubules (Figure 2S and Table 4C). This frequency was similar to the 70% misoriented microtubules observed in the equivalent class of *kar9Δ* cells (Miller and Rose, 1998). While the *kip3Δ* microtubule orientation defect is similar to that observed for the *kar9Δ* mutant, abnormally long cytoplasmic microtubules were not observed in *kar9Δ* strains (Kurihara *et al.*, 1994; Miller and Rose, 1998). In contrast to *kip3Δ*, the *dhc1Δ* mutants nearly always contained a cytoplasmic bundle that extended into the bud, even in

Table 2. Plasmids used in this study

Strain	Genotype	Source
pMR3144	<i>KIP2 CEN URA3</i>	Leibler
pMR3465	<i>P_{GAL}-GFP-KAR9 LEU2 CEN4 ARS1</i>	
pMR3769	<i>GFP#2</i>	
pMR3777	<i>KIP2::HA CEN URA3</i>	
pMR3778	<i>KIP2 2μ URA3</i>	Broach
pMR3779	<i>KIP2::HA 2μ URA3</i>	
pB1893	<i>P_{GALT}-GFP CEN4 ARS1 LEU2</i>	
pMR3635	<i>P_{GALT}-GFP-KIP3 CEN4 ARS1 LEU2</i>	
pMR3889	<i>P_{GALT}-GFP-KIP2 CEN URA3</i>	Hieter
pRS403	<i>Yip-HIS3</i>	
pRS426	<i>2μ URA3</i>	Hieter
pTRP17-20	<i>act5Δ1-366::TRP1</i> disruption plasmid	Clark
pJM1432	<i>jnm1::LEU2</i> disruption plasmid	Tatchell
pBR2-1U	<i>dhc1::URA3</i> disruption plasmid	Bloom
pVB17	<i>bik1::TRP1</i> disruption plasmid	Fink
pGTEP1	Triple HA epitope	Futcher

Unless indicated otherwise, all plasmids are from this laboratory.

Figure 1. DAPI staining of abnormal nuclear morphologies in *kip2Δ* and *kip3Δ* mutants. The *kip2Δ* strain, MS2309 (A–D), and the *kip3Δ* strain, MS4516 (E–H), were grown to early exponential phase in YPD medium at either 14°C (MS2309) or 12°C (MS4516) and stained with DAPI to visualize the nuclear material.

aberrant cells exhibiting nuclear positioning defects (Table 4, B and C) in agreement with previous reports (Li *et al.*, 1993).

To summarize the microtubule morphology data, both *kip2Δ* and *kip3Δ* influence the length of the cytoplasmic microtubules but appear to have opposite effects. By inference, Kip2p seems to be required to stabilize or assemble the cytoplasmic microtubules, whereas Kip3p appears to destabilize them. In addition, Kip3p may have a second function that is important for the orientation of cytoplasmic microtubules.

Genetic Analysis of *kip2Δ* Mutants Places Kip2p in the Dynein/Dynactin Pathway of Nuclear Positioning

Our previous work showed that *DHC1* and *KAR9* function in partially redundant pathways for nuclear migration (Miller and Rose, 1998). Because the mitotic defects observed in the *kip2Δ* and *kip3Δ* mutants were similar to those seen in *dhc1Δ* and *kar9Δ* mutants, we wanted to determine whether Kip2p and Kip3p might function in either of these two pathways. To carry out the analysis, we performed crosses with strains bearing deletions in each of the kinesin-related genes along with several other relevant genes required for spindle function and/or nuclear positioning. The viability and growth characteristics of the resulting double-mutant strains were then used as an assay for possible genetic interactions. Table 5 summarizes the results from the crosses, and Figure 3 depicts a typical cross in which a synthetic growth defect was apparent.

First, the *kip2Δ* mutant was crossed to the mutants that exhibit defects in nuclear migration (Table 5, top panel). We found that all *kip2Δ kar9Δ* double mutants were inviable, or synthetically lethal (Figure 3 and Table 5, line 1), suggesting that Kip2p and Kar9p act in separate pathways for nuclear migration. Cells from the *kip2Δ kar9Δ* microcolonies exhibited a severe defect in nuclear migration; many were multinucleated and had anucleate buds (Figure 3B). In contrast, double mutants with *kip2Δ* in combination with deletions in genes of the dynein/dynactin complex (*dhc1Δ*, *jnm1Δ* and *act5Δ*) were viable and displayed no visible differences in growth compared with wild type (Table 5, lines 2, 3, and 4). Furthermore, all *kip2Δ dhc1Δ kar9Δ* triple mutants were indistinguishable in size from the *kip2Δ kar9Δ* or *dhc1Δ kar9Δ* double mutants (Table 5, line 19). Taking the single, double, and triple mutant analyses together, the data suggest that Kip2p functions in the dynein/dynactin branch of the nuclear migration pathway and not in a third redundant pathway.

Genetic Interactions Between *kip2Δ* and Mutations Affecting Microtubule Function

BIK1 encodes a microtubule-associated protein that, like Kip2p, is thought to stabilize microtubules and also functions in nuclear positioning in mitosis and mating (Berlin *et al.*, 1990). Given these phenotypes, we wanted to determine whether there are genetic interactions between *kip2Δ* and *bik1Δ*. All of the *kip2Δ bik1Δ* double mutants were viable (Table 5, line 5). These results are consistent with the hypothesis that Kip2p and Bik1p might stabilize microtubules through a common pathway. In support of this, mutations in *bik1* show synthetic lethality with mutations in *TUB2*, the gene for β -tubulin (Berlin *et al.*, 1990), and the *kip2Δ* mutation was found to be

Table 3. Nuclear migration defects of *kip2Δ* and *kip3Δ* mutants

A <i>kip2Δ</i>						
Strain	°C	n				Σ % Abnormal
wild type	30	309	0.3	1.3	0.7	2.3
<i>kip2Δ ::TRP1</i>	30	252	0.4	5.2	2.0	7.5
<i>kip2Δ ::URA3</i>	30	264	1.5	4.2	0.8	6.4
wild type	12	402	0.4	1.7	0.4	2.1
<i>kip2Δ ::TRP1</i>	12	269	2.2	3.3	16.0	21.0
<i>kip2Δ ::URA3</i>	12	333	3.9	5.1	9.3	18.0

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Figure 2. Microtubule morphologies of *kip2Δ* and *kip3Δ* mutants. The wild-type strain, MS1554 (A–C), the *kip2Δ* strain, MS2309 (D–L), and the *kip3Δ* strain, MS4516 (M–U), were grown to early exponential phase at 14°C. Cells were fixed in formaldehyde and stained with anti-tubulin YOL1/34 antibody (A, D, G, J, M, P, and S). Cells were also stained with DAPI to visualize nuclear material (B, E, H, K, N, Q, and T). Visualization of cells by Nomarski optics is seen in panels C, F, I, L, O, R, and U.

synthetically lethal in combination with mutations in *TUB1* (our unpublished observations).

Given the precedence of functional redundancy between kinesin-related proteins (Roof *et al.*, 1992; Saunders and Hoyt, 1992), we next performed crosses between *kip2Δ* and deletions of the other kinesin-related genes. The kinesin-related gene *SMY1* appears to act in concert with a myosin, Myo2p, in the secretory pathway (Lillie and Brown, 1992). In crosses, the *kip2Δ smy1Δ* double mutants were viable (Table 5, line 9). Genetic interactions with *cin8Δ*, a kinesin-related gene known to function in mitosis, were also performed. All *kip2Δ cin8Δ* double mutants were inviable (Table 5, line 6). Previous work showed that *kip2Δ* is not synthetically lethal with a deletion of *KIP1* or with a deletion of *KAR3*, two other mitotic kinesin-related genes (Roof *et al.*, 1992). Finally, we crossed the *kip3Δ* mutation and the *kip2Δ* mutation. All *kip2Δ kip3Δ* double mutants were viable and exhibited normal growth. In summary, in genetic crosses with all of the known kinesin-related genes, *kip2Δ* was found to be synthetically lethal only with *cin8Δ*.

Genetic Analysis of kip3Δ Mutants Places Kip3p in the KAR9 Pathway of Nuclear Positioning

To understand the functions of KIP3 in nuclear migration, crosses to *kar9Δ*, *dhc1Δ*, and other mutations affecting nuclear migration were performed. We found that 88% of the *kip3Δ kar9Δ* double mutants were viable (Table 5, line 10). Thus, *kip3Δ*, unlike *kip2Δ*, was not more severe when combined with *kar9Δ*. Furthermore, *kip3Δ* resulted in severe growth defects in combination with mutations in genes in the dynein/dynactin complex. In crosses to *dhc1Δ*, *jnm1Δ* and *act5Δ*, all of the predicted double mutants were dead or produced microcolonies (Table 5, lines 11–13). Cells from the *kip3Δ dhc1Δ* microcolonies exhibited an extreme nuclear migration defect; many were multinucleated, some had fragmented nuclei, and nearly 25% had lysed. These results suggest that the *kip3Δ* defect interferes with a pathway that is partially redundant with the dynein–dynactin pathway for nuclear migration. Furthermore, we found that all *kip3Δ dhc1Δ kar9Δ* triple mutants were indistinguishable from the *kip3Δ dhc1Δ* and *dhc1Δ kar9Δ* double mutants (Table 5, line 20). Taking the single, double, and triple mutant data together, we conclude that Kip3p functions in the KAR9 branch of the nuclear migration pathway and not in a third redundant pathway.

Finally, we examined the *kip2Δ kip3Δ kar9Δ* triple mutant and found that it was inviable (Table 5, line 21), unlike the *kip2Δ kip3Δ* double mutant. This result suggests that, although Kar9p and Kip3p act in the same pathway, Kar9p must still be active in the *kip2Δ kip3Δ* double mutant.

Table 4. Quantification of microtubule morphologies

A Telophase
		medium or short cMTs	no cMTs	long cMTs	disassembled spindle
Strain:					
wild type	n=74	79	14	0	8
<i>dhc1Δ</i>	n=65	59	22	2	18
<i>kip2Δ</i>	n=68	41	53	0	6
<i>kip3Δ</i>	n=85	50	5	35	9

B Abnormal Anaphase
		cMTs extending into bud	cMTs not extending into bud	no cMTs	long cMTs
Strain:					
wild type	n=53	68	4	28	0
<i>dhc1Δ</i>	n=48	77	0	23	0
<i>kip2Δ</i>	n=61	18	0	82	0
<i>kip3Δ</i>	n=55	46	24	27	2

Table 4. (continued)

C Telophase in Mother
		cMTs extending into bud	cMTs not extending into bud	no cMTs	disassembled spindle
Strain:					
<i>dhc1Δ</i>	n=72	83	4	1	11
<i>kip2Δ</i>	n=71	21	4	70	4
<i>kip3Δ</i>	n=86	17	72	5	4

The wild-type strain (MS1554), the *kip2Δ* strain (MS2309), the *kip3Δ* strain (MS4516), and the *dhc1Δ* strain (MS4903) were grown to early exponential phase at 14°C and fixed for indirect immunofluorescence with anti-tubulin (see MATERIALS AND METHODS). The data for the wild-type and *dhc1Δ* controls were also reported by Miller and Rose (1998).

Genetic Interactions Between *kip3* and Mutations Affecting Microtubule Function

The *kip3Δ* mutation was also tested in combination with mutations in genes known to affect microtubule stability and/or spindle function. All *kip3Δ kip1Δ* double mutants were viable (Table 5, line 16). In contrast, all of the *kip3Δ bik1Δ* (Table 5, line 14), *kip3Δ kar3Δ* (Table 5, line 17), and most of the *kip3Δ cin8Δ* (Table 5, line 15) double mutants were inviable. Therefore, *kip3Δ* mutants exhibited severe growth defects in combination with *kar3Δ*, *cin8Δ*, and *bik1Δ* mutants, but not *kip1Δ* mutants. Thus, *kip2Δ* and *kip3Δ* differed with respect to their interactions with *bik1Δ* and *kar3Δ*, but were similar with respect to their interactions with *kip1Δ* and *cin8Δ*.

It is striking that the two mutations that primarily affect spindle elongation, *cin8Δ* and *kip1Δ*, do not produce distinct genetic interactions with *kip2Δ* and *kip3Δ*. One interpretation of this observation is that any strong defects in spindle elongation will be synthetically lethal with any mutation that causes strong defects in spindle orientation, regardless of the mechanism. In contrast, *Bik1p* and *Kar3p* both have significant effects on the cytoplasmic microtubules and

show differential interactions with *kip2Δ* and *kip3Δ*. It seems likely that the specificity of the interaction reflects their quite different roles in cytoplasmic microtubule function.

kip2 Mutants Show Altered Cellular Localization of *Kar9p*

In support of the hypothesis that *Kip2p* and *Kip3p* function differently in nuclear migration, we found that localization of GFP-*Kar9p* was altered in a *kip2Δ*, but not in *kip3Δ* or *dhc1Δ* mutants (Figure 4). As was reported previously (Miller and Rose, 1998), in a majority of wild-type preanaphase cells, GFP-*Kar9p* localized exclusively to one or two dots (41 and 26%, respectively) at the cortex of the bud (Figure 4). In a minority of wild-type cells (22%), in addition to the cortical localization, a second spot was observed on the edge of the nucleus close to the bud neck bud, presumably associated with the SPB. For both *kip3Δ* and *dhc1Δ*, the pattern of GFP-*Kar9p* localization was indistinguishable from wild type. In contrast, in the *kip2Δ*, GFP-*Kar9p* was exclusively localized to the bud tip cortex in less than 2% of the cells. In most *kip2Δ*

Table 5. Viability of double mutants in combination with *kip2Δ* or *kip3Δ*

Mutant combination	Tetrads analyzed	No. of predicted mutants	% of mutants dead or forming microcolonies	Viability of double or triple mutant
1. <i>kip2Δ kar9Δ</i>	33	33	100	SL
2. <i>kip2Δ dhc1Δ</i>	33	31	6	viable
3. <i>kip2Δ jnm1Δ</i>	11	10	0	viable
4. <i>kip2Δ act5Δ</i>	12	13	0	viable
5. <i>kip2Δ bik1Δ</i>	10	11	0	viable ^a
6. <i>kip2Δ cin8Δ</i>	21	18	100 ^b	SL
7. <i>kip2Δ kip1Δ</i>	—	—	—	viable ^c
8. <i>kip2Δ kar3Δ</i>	—	—	—	viable ^c
9. <i>kip2Δ smy1Δ</i>	8	7	0	viable
10. <i>kip3Δ kar9Δ</i>	29	23	12	viable
11. <i>kip3Δ dhc1Δ</i>	16	15	100	SL
12. <i>kip3Δ jnm1Δ</i>	29	29	100	SL
13. <i>kip3Δ act5Δ</i>	13	9	100	SL
14. <i>kip3Δ bik1Δ</i>	13	12	100	SL
15. <i>kip3Δ cin8Δ</i>	18	24	75 ^d	SL
16. <i>kip3Δ kip1Δ</i>	9	9	0	viable
17. <i>kip3Δ kar3Δ</i>	17	13	100	SL
18. <i>kip3Δ kip2Δ</i>	17	14	0	viable
19. <i>kip2Δ dhc1Δ kar9Δ</i>	18	8	100	SL
20. <i>kip3Δ dhc1Δ kar9Δ</i>	12	5	100	SL
21. <i>kip2Δ kip3Δ kar9Δ</i>	54	19	100	SL

Standard meiotic crosses were used to construct the indicated double mutants. The size of colonies was scored 2-3 d after germination. SL indicates that synthetic lethality was observed. The following crosses were carried out to create the indicated double and triple mutants: 1. *kip2Δ kar9Δ*, MS2310 × MS4263; 2. *kip2Δ dhc1Δ*, MS2354 × MS4304; 3. *kip2Δ jnm1Δ*, MS2310 × MS4321; 4. *kip2Δ act5Δ*, MS4308 × MS4586; 5. *kip2Δ bik1Δ*, MS4308 × MS4734; 6. *kip2Δ cin8Δ*, MS2310 × MAY2059; 9. *kip2Δ smy1Δ*, APY4ΔD5 × MS2354; 10. *kip3Δ kar9Δ*, MS4516 × MS4316; 11. *kip3Δ dhc1Δ*, MS4516 × MS4600; 12. *kip3Δ jnm1Δ*, MS4692 × MS4516; 13. *kip3Δ act5Δ*, MS4691 × MS4701; 14. *kip3Δ bik1Δ*, MS4691 × MS4915; 15. *kip3Δ cin8Δ*, MS4516 × MAY2058; 16. *kip3Δ kip1Δ*, MS4516 × MS2305; 17. *kip3Δ kar3Δ*, MS4516 × MS660; 18. *kip3Δ kip2Δ*, MS2442 × MS4691; 19. *kip2Δ dhc1Δ kar9Δ*, MS5214 × MS4062; 20. *kip3Δ dhc1Δ kar9Δ*, MS4654 × MS4304; and 21. *kip2Δ kip3Δ kar9Δ*, MS4308 × MS4648.

^a Identical results were observed when *kip2Δ* was crossed to a *bik1Δ* in a nonisogenic strain (our unpublished observations).
^b The viability of *cin8Δ* single mutants in this cross was 52%. This double mutant was also examined by plasmid loss assay on 5-FOA using a *KIP2 URA3 CEN* plasmid, which demonstrated that the *cin8Δ kip2Δ* double mutant lost a *KIP2 CEN* plasmid much less frequently than either wild-type or either single mutant.
^c Data from Roof *et al.* (1992).
^d The viability of *cin8Δ* single mutants in this cross was 67%.

cells (85%), GFP-Kar9p localized both to the tip of the bud and to edge of the nucleus very close to the bud neck (Figure 4).

Kip2p and Kip3p Show Distinct Cellular Localization Patterns

To further define the roles of these kinesin-related proteins in mitosis, we next determined their intracellular location. For visualization of Kip2p, we generated two different tagged protein constructs, GFP-Kip2p and Kip2p-HA. Expression of either construct suppressed the growth defects of *kip2Δ kar9Δ* double mutants, demonstrating that the proteins were functional. Visualization of GFP-Kip2p using fluorescence microscopy (Figure 5, A–H) and Kip2p-HA by indirect immunofluorescence microscopy (Figure 5I) showed

that Kip2p localized exclusively on cytoplasmic microtubules. At all stages of the cell cycle, GFP-Kip2p fluorescence corresponded to the cytoplasmic microtubule pattern (Figure 5). Furthermore, Kip2p localization was observed on the cytoplasmic microtubules in both the mother and the bud. Localization to the nuclear microtubules was not detected. The localization pattern was identical in *kip2Δ* and *KIP2* strains. Kip3p localization was determined using a *P_{GAL1}-GFP-KIP3* construct transformed into a wild-type diploid (MS4720). Early in the cell cycle in unbudded and small-budded cells, GFP-Kip3p localized to the SPB and the associated cytoplasmic microtubules (Figure 6A). After spindle formation, GFP-Kip3p fluorescence was associated with the SPBs, the spindle, and the cytoplasmic microtubules (Figure 6, D–J). Less than

5% of cells appeared to have no GFP-Kip3p signal, but this was not specific to any particular cell-cycle stage. Identical localization patterns were found using GFP-KIP3 in the homozygous *kip3Δ* omit strain.

Many of the cells overexpressing GFP-KIP3 showed abnormal morphologies. Some cells were arrested as large-budded cells with a single nucleus that had not elongated or migrated to the bud neck. This phenotype was noted in 41% of large-budded cells when expressed in the wild-type strain, and in 50% when expressed in the *kip3Δ* strain MS4719. Because of the abnormal overexpression phenotypes, it was not possible to determine whether the GFP-KIP3 expressed functional Kip3p.

In summary, although both proteins show localization patterns consistent with microtubule structures, Kip2p appears to localize exclusively to cytoplasmic microtubules, whereas Kip3p is found on both the spindle and cytoplasmic microtubules. The results from the localization further confirm that the two proteins play distinct roles in mitosis and nuclear positioning.

DISCUSSION

In this study, we confirm that the kinesin-related genes, *KIP2* and *KIP3*, are involved in yeast nuclear migration during mitosis (Miller and Rose, 1995; Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). Consistent with defects in nuclear migration, the cytoplasmic microtubules were aberrant in *kip2Δ* and *kip3Δ* strains. As was found by others (Cottingham and Hoyt, 1997), in *kip2Δ* strains the cytoplasmic microtubules were absent or dramatically shorter than wild type. In contrast, we found that in *kip3Δ* strains the cytoplasmic microtubules were present but misoriented and often failed to enter the bud. Moreover, we confirmed that the cytoplasmic microtubules were much longer than in wild type (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). In our study this was most

pronounced at telophase. Genetic analysis showed that Kip2p and Kip3p function very differently in nuclear migration. Specifically, mutations in the two genes had complementary patterns of synthetic lethality when combined with mutations in genes that affect cytoplasmic microtubule function. Extending earlier observations, *kip2Δ* was synthetically lethal in combination with *kar9Δ*, but was viable in combination with *dhc1Δ*, *jnm1Δ*, and *act5Δ*. In contrast, *kip3Δ* was viable in combination with *kar9Δ* mutations but was synthetically lethal when combined with *dhc1Δ*, *jnm1Δ*, and *act5Δ*. These genetic results suggest that Kip2p functions as part of the dynein pathway for nuclear migration, whereas Kip3p functions as part of the Kar9p pathway. Localization studies also showed that Kip2p and Kip3p function differently in mitosis. Kip2p localized exclusively to the cytoplasmic microtubules, whereas we and others (DeZwaan *et al.*, 1997) found that Kip3p was present on both spindle and cytoplasmic microtubules. Taken together, these data support a model of nuclear migration in which Kip2p-dependent microtubule stability is required for dynein-dependent nuclear movement, whereas Kip3p is required for the cytoplasmic microtubule orientation function of Kar9p.

kip2Δ and *kip3Δ* Mutants Display Distinct Cytoplasmic Microtubule Morphologies

The morphology of the microtubules in the *kip2Δ* strain suggest that wild-type Kip2p plays a role in stabilizing the cytoplasmic microtubules. Consistent with this, we observed that overexpression of Kip2p on a 2 μ plasmid resulted in 10% of the cells exhibiting hyperelongated microtubules that looped around the inside of the cells. In addition, increased overexpression using a galactose-inducible promoter was toxic to cells (our unpublished observations).

In contrast to *kip2Δ*, the *kip3Δ* mutants displayed normal cytoplasmic microtubules during the early stages of

Figure 3. A representative cross exhibiting synthetic lethality. Diploids resulting from the cross between *kip2Δ* and *kar9Δ* were sporulated, and the tetrads were dissected on YPD plates. After 2–3 d of growth at 30°C, the colonies were scored for normal growth, microcolonies, or no growth. A dissection plate after 2 d is shown in panel A. The small arrows indicate two *kip2Δ kar9Δ* microcolonies. Several other microcolonies became apparent after prolonged incubation. Panel B shows a typical multinucleate mother cell with several anucleate buds from a *kip2Δ kar9Δ* microcolony. The cells were stained with the fluorescent DNA-specific dye, DAPI, to allow visualization of the nuclei, and photographed using fluorescence and differential interference contrast optics simultaneously.

Figure 4. GFP-Kar9p localization in nuclear migration mutants. Representative cells show the pattern of fluorescence of GFP-Kar9p (right) and the corresponding nuclear and cellular morphologies (left). The two left-most panels are a cell with GFP-Kar9p at the tip of the bud (single-dot cortex). The middle panels are of a cell with GFP-Kar9p at the tip of the bud and elsewhere in the bud (two dots, cortex and in bud). The right-most panels show a cell with GFP-Kar9p at the tip of the bud in and at the nuclear periphery (two dots, cortex and bud neck/SPB). The wild-type (WT), (*MS1554*), *kip3Δ* (*MS4516*), *dhc1Δ* (*MS5001*), and *kip2Δ* (*MS2309*) strains were transformed with a *GFP-KAR9* (*pMR3465*) construct and scored for GFP-Kar9p localization in preanaphase cells (medium-budded cells with a single nucleus). Between 242 and 508 cells were counted for each strain. For the various strains, percentages of total cells with the localization patterns are shown under the representative cell. Between 3 and 6% of the cells showed no fluorescence, and between 6 and 10% showed patterns that did not fall into the three classes represented in the figure.

mitosis. However, at telophase the cytoplasmic microtubules were often significantly longer than normal. By inference, these observations suggest that Kip3p destabilizes the cytoplasmic microtubules during at least one period in mitosis. In support of the suggestion that Kip3p shortens the microtubules, overexpression of Kip3p-GFP appeared to diminish the cytoplasmic microtubules during anaphase (Figure 6, G–L).

How might proteins that are thought to function in microtubule-dependent movements affect the stability/assembly of the microtubules in opposite ways? First, it is possible that a kinesin-related protein may not act as a motor, but may instead bind in a stable manner to microtubules. As such, a kinesin-related protein could help stabilize a microtubule against depolymerization. For example, mutant forms of Kar3p (*kar3-1* and Kar3p- β -galactosidase) that cannot hydrolyze ATP do stabilize the cytoplasmic microtubules during mating (Meluh and Rose, 1990; Vallen *et al.*, 1992). Second, some kinesins, such as Kar3p and XKCM1, have the intrinsic ability to depolymerize microtubules (Endow *et al.*, 1994; Walczak *et al.*, 1996). Finally, in the absence of ATP, kinesin and kinesin-related proteins can “track” along the ends of microtubules as they depolymerize (Coue *et al.*, 1991; Lombillo *et al.*, 1995). Indeed, the sliding attachment can

couple depolymerization to the movement of attached organelles, effectively converting disassembly into work. It seems reasonable to propose that the association of kinesin-related proteins with the depolymerizing ends of microtubules would have significant effects on the stability of microtubules, with variable and distinct effects depending on the properties of the specific protein.

Kip2p and Kip3p Have Different Roles in Nuclear Migration

Broadly speaking, dynein and Kar9p define two partially redundant pathways for efficient nuclear migration; the former largely providing the force for movement and the latter largely providing the orientation. It is striking that the genetic analysis indicated that *kip2Δ* and *kip3Δ* each specifically affect the dynein and Kar9p pathways, respectively. The complementary nature of the genetic interactions provides important clues as to their roles in nuclear movement.

One interpretation of our finding that *kip2Δ* affects the dynein pathway is that Kip2p interacts directly with dynein or the proteins in the dynactin complex. Although native cytoplasmic dynein has not yet been localized in yeast, a functional GFP-dynein hybrid local-

Figure 5. Kip2p localization. To localize GFP-Kip2p, a *kip2Δ* strain (MS2354) containing the GFP-Kip2 plasmid (pMR3889) was grown to early exponential phase, formaldehyde fixed, and stained with DAPI. Left panels are fluorescence, middle panels are DAPI staining, and right panels are Nomarski images of the same cell. Representative cells at different stages of the cell cycle are shown in the

izes along the length of the cytoplasmic microtubules (Shaw *et al.*, 1997). The similar localization of Kip2p-GFP and Kip2p-HA along the length of the cytoplasmic microtubules is consistent with the suggestion that the two proteins interact. However, there is no biochemical evidence to support a direct interaction. One possible role for Kip2p would be as a transporter to move dynein back toward the plus ends of the microtubules. One explicit prediction of this hypothesis is that the localization of Kip2p and dynein along the cytoplasmic microtubules would be interdependent.

However, the fact that dynein does not appear to be restricted to a cortical site raises the possibility that dynein acts to produce force over the entire length of the cytoplasmic microtubules. Presumably, dynein would also interact with other cytoskeletal elements to provide the counterbalance for force production. Assuming that dynein does act over the entire length of the microtubules, the total force exerted by dynein would be directly related to the total length of the cytoplasmic microtubules. Accordingly, any mutation that leads to destabilization or failure to assemble the cytoplasmic microtubules would also compromise dynein-dependent force production. Thus, Kip2p would genetically appear to be a member of the dynein pathway because of the reduced length of the cytoplasmic microtubules and not because of any specific association between dynein and Kip2p. In support of this view, we observed that mutations in *BIK1*, which also destabilize the cytoplasmic microtubules, were synthetically lethal with *kip3Δ* and *kar9Δ* (Miller and Rose, 1998) but not with *kip2Δ*. The viability of the *bik1Δ kip2Δ* double mutant suggests that the microtubules may not be significantly more destabilized than in either single mutant.

The synthetic lethality of *kip2Δ* with *kar9Δ* demonstrates that Kar9p is still at least partially active in the *kip2Δ* mutant. Unlike dynein, GFP-Kar9p localized to a cortical site at the ends of the cytoplasmic microtubules (Miller and Rose, 1998). Accordingly, Kar9p's function should be much less dependent upon microtubule length. Microtubules undergoing dynamic growth would become long enough to interact with Kar9p at the cortex, and Kar9p would then be able to stabilize and orient them. It is important to note that indirect immunofluorescence of cytoplasmic microtubules in fixed cells underestimates both their length and number (Carminati and Stearns, 1997). Thus, the

Figure 5 (cont). figure. (A) Unbudded or G₁ cell, n = 89; (B and C) small budded, n = 97; (D and E) medium budded, n = 107; (F-H) large budded, n = 93. The bottom panels (I) show indirect immunofluorescence of HA on the left, DAPI staining in the middle, and Nomarski image on the right. To localize Kip2p using an HA epitope, a *kip2Δ* strain (MS2354) containing pMR3779 (a Kip2p::HA 2μ construct) was grown to early exponential phase and paraformaldehyde fixed (see MATERIALS AND METHODS).

cytoplasmic microtubules in the *kip2Δ* mutant may be longer and more abundant in live cells.

It is striking that the Kar9-GFP showed a significantly different localization in *kip2Δ* compared with wild type. While localization of Kar9p to the cortex does not require microtubules (Miller and Rose, 1998), the effect of *kip2Δ* implies that microtubules can nevertheless influence its localization. The secondary localization of Kar9p to the nuclear periphery/bud neck region in *kip2Δ* mutants might allow the short microtubules to aid in nuclear migration by interacting with Kar9p near the bud neck.

The model that dynein and Kar9p function on different sites of the cytoplasmic microtubules predicts that more effective microtubule-destabilizing mutants would have a much more severe defect in nuclear migration than *kar9Δ* or *dhc1Δ*. The absence of cytoplasmic microtubules would necessarily inactivate both pathways for nuclear migration. This is precisely what was observed for *tub2-401* and other *tub2* mutations under conditions that specifically depolymerize the cytoplasmic microtubules (Sullivan and Huffaker, 1992).

In contrast to *KIP2*, our analysis of *KIP3* mutations suggested that Kip3p acts in the Kar9p pathway. Given GFP-Kip3p's localization along the length of both the nuclear and cytoplasmic microtubules, Kar9p's localization at the ends of the cytoplasmic microtubules (Miller and Rose, 1998) would seem to preclude a simple model of Kar9p–Kip3p interaction. One explanation for the genetic data would be that Kar9p does not interact directly with microtubules, but instead interacts with Kip3p on the microtubules. According to this view, Kip3p would appear to have more than one function in the cell. One function for Kip3p would be to destabilize or depolymerize microtubules via interactions at various sites within the cell. A second function would be for Kip3p to serve as a microtubule “adapter” for Kar9p. This model predicts that Kar9p's association with microtubules would be completely dependent on Kip3p and that *kip3Δ* mutants would be effectively Kar9[−].

While the “adapter” model is superficially attractive, the two mutations act differently in two important respects. First, whereas *kip2Δ* is synthetically lethal with *kar9Δ*, *kip2Δ* is not synthetically lethal with *kip3Δ*. Second, although *kip2Δ* mutations suppress the synthetic lethality of *kip3Δ dhc1Δ* double mutants (Cottingham and Hoyt, 1997), *kip2Δ* does not suppress the synthetic lethality of *kar9Δ dhc1Δ* double mutants. Therefore, although both *kar9Δ* and *kip3Δ* have similar defects with respect to microtubule orientation, they cannot be said to be equivalent. By inference, *kip3Δ* mutants must be effectively Kar9⁺. In support of this, we found that the *kip2Δ kip3Δ kar9Δ* triple mutant was inviable. Furthermore, we found that Kar9p localization in the *kip3Δ* was identical to wild type. An alternative model for the *kip3Δ* defect in nuclear migration

Figure 6. GFP-Kip3p localization. To localize GFP-Kip3p, a wild-type strain (MS4720) containing the GFP-Kip3p plasmid (pMR3635) was grown at 30°C, formaldehyde fixed, and DAPI stained. GFP-Kip3p localization is shown in panels A, D, G, and J. DAPI staining is shown in panels B, E, H, and K. Nomarski images are shown in C, F, I, and L.

would unify the two apparent functions for Kip3p, microtubule destabilization and orientation. Theoretical analysis of microtubule dynamics has demonstrated that the time required for microtubules to find their targets in space is critically dependent upon dynamic instability (Holy and Leibler, 1994). Computer simulations demonstrated that nondynamic microtubules take several orders of magnitude longer time to find distal targets. According to this model, the *kip3Δ* would cause a defect in microtubule orientation as a consequence of the increased length and presumably increased stability of the cytoplasmic microtubules. In the *kip3Δ* mutant, Kar9p would still be functional but unable to interact with the persistently misoriented microtubules. Accordingly, *kip3Δ* would mimic the *kar9Δ* with respect to the microtubule orientation defect and show lethality with mutations affecting dynein and dynactin subunits.

A complication for the model for nuclear migration described above concerns the viability of the *kip2Δ kip3Δ* double mutant. The simple expectation from their patterns of genetic interactions is that they should also exhibit synthetic lethality. However, we and others found that the two deletion mutations have opposite effects on microtubule length (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). Furthermore the two mutations show several compensatory effects in mitosis. In particular, *kip3Δ* suppresses the benomyl sensitivity of *kip2Δ* mutants (Cottingham and Hoyt, 1997). Therefore, it is likely that the viability of the double mutant occurs because of the compensatory effects of the two mutations on microtubule stability. In one scenario, *kip2Δ* would shorten the hyperelongated microtubules observed in *kip3Δ*, allowing them to interact with Kar9p. From this argument, the inviability of the *bik1Δ kip3Δ* double mutant becomes somewhat less clear. However, *bik1Δ* may have a more severe defect for cytoplasmic microtubule stability than *kip2Δ*, or *bik1Δ* may affect the nuclear microtubules as well as the cytoplasmic microtubules (Berlin *et al.*, 1990).

Genetic Interactions with Spindle-Elongation Mutations

In our study, both *kip2Δ* and *kip3Δ* mutations were found to be synthetically lethal with *cin8Δ* but not with *kip1Δ*. Cin8p and Kip1p play partially redundant functions in spindle assembly and maintenance (Hoyt *et al.*, 1992; Roof *et al.*, 1992). However, Cin8p appears to play a larger role for spindle function because mutations in *CIN8* lead to a conditional growth defect, whereas mutations in *KIP1* do not. Previous reports demonstrating that dynein deletions are synthetically lethal with *CIN8* deletions have been interpreted to mean that dynein can provide part of the force for spindle elongation, presumably by acting on the cytoplasmic microtubules (Saunders *et al.*, 1995). Our finding of lethality for the *cin8Δ kip2Δ* double mutant (also observed by Cottingham and Hoyt, 1997) is consistent with this hypothesis. By the same argument, the lack of synthetic lethality between the *CIN8* deletion and the *KAR9* deletion (Miller and Rose, 1998) implies that the orientation function of Kar9p is not required for the redundant force that powers spindle elongation in the *cin8Δ*. Consequently, our finding of lethality for the *kip3Δ cin8Δ* double mutant was initially surprising. However, Kip3p clearly has functions that Kar9p does not, which can be discerned in the *kip3Δ* mutant by the hyperelongated microtubules. Furthermore, the fact that Kip3p-GFP localizes to both nuclear and cytoplasmic microtubules suggests that Kip3p participates in spindle function as well as nuclear migration. Thus, it seems more likely that the synthetic lethality of the *kip3Δ cin8Δ* double mutant arises from defects in spindle function rather than nuclear movement. We

note that synthetic lethality of *kip3Δ cin8Δ* was not observed by DeZwaan *et al.*, (1997). The basis for this difference in our results is unclear at present. Finally, *kip2Δ* and *kip3Δ* also differed in their interaction with *kar3Δ*; *kip2Δ kar3Δ* was viable whereas *kip3Δ kar3Δ* was inviable (our present results and Roof *et al.*, 1992; Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). While the exact function of Kar3p in mitosis remains unclear, it appears both to antagonize the function of Cin8p and Kip1p and promote microtubule disassembly (Saunders and Hoyt, 1992; Endow *et al.*, 1994; Saunders *et al.*, 1997). It is striking that both Kip3p and Kar3p appear to destabilize microtubules; this suggests that their synthetic defect might arise from a combined and catastrophic defect in microtubule disassembly.

Conclusion

Three lines of investigation, microtubule morphology, genetic interactions, and localization of the proteins, support the hypothesis that Kip2p and Kip3p function in nuclear migration by two different pathways. Our data strongly suggest that Kip2p function is required for normal dynein-dependent movement, whereas Kip3p function is required for Kar9p-dependent cytoplasmic microtubule orientation.

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