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Mitotic Spindle Function in *Saccharomyces cerevisiae* Requires a Balance between Different Types of Kinesin-related Motors

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Two *Saccharomyces cerevisiae* kinesin-related motors, Cin8p and Kip1p, perform an essential role in the separation of spindle poles during spindle assembly and a major role in spindle elongation. Cin8p and Kip1p are also required to prevent an inward spindle collapse prior to anaphase. A third kinesin-related motor, Kar3p, may act antagonistically to Cin8p and Kip1p since loss of Kar3p partially suppresses the spindle collapse in *cin8 kip1* mutants. We have tested the relationship between Cin8p and Kar3p by overexpressing both motors using the inducible *GAL1* promoter. Overexpression of *KAR3* results in a shrinkage of spindle size and a temperature-dependent inhibition of the growth of wild-type cells. Excess Kar3p has a stronger inhibitory effect on the growth of *cin8 kip1* mutants and can completely block anaphase spindle elongation in these cells. In contrast, overexpression of *CIN8* leads to premature spindle elongation in all cells tested. This is the first direct demonstration of antagonistic motors acting on the intact spindle and suggests that spindle length is determined by the relative activity of Kar3p-like and Cin8p/Kip1p-like motors.

INTRODUCTION

During eukaryotic cell division, the microtubule-based spindle is responsible for the segregation of both the microtubule organizing center and the attached chromosomes. During mitosis, the microtubule organizing center becomes the spindle poles, and movement of the poles can be a good indicator of force production within the spindle. As with many other forms of intracellular transport, separation of the spindle poles is a consequence of the activity of microtubule-associated force-generating motor proteins.

In the budding yeast, *Saccharomyces cerevisiae*, four motor proteins have been identified which play defined roles in the separation of the spindle poles. Cytoplasmic dynein apparently acts on the cytoplasmic microtubules to accurately position the spindle near the neck separating the mother and bud (Yeh *et al.*, 1995) and later in anaphase to separate the two spindle poles (Saunders *et al.*, 1995). Two kinesin-related motors, Cin8p and Kip1p, also function in the separation of the spindle poles. These proteins perform a partially redundant role early in mitosis during spindle assembly (Hoyt *et al.*, 1992; Roof *et al.*, 1992) and remain required for spindle pole separation through the onset of anaphase (Saunders and Hoyt, 1992). Both Cin8p and Kip1p are located on the intranuclear microtubules within the spindle and most likely act to push out on the spindle poles. Cin8p and Kip1p are the *S. cerevisiae* representatives of the *bimC* family of spindle motors (Enos and Morris, 1990). The sequence of *bimC*-like motors are not well conserved outside of the common motor domain, but their function seems to be well conserved between diverse eukaryotic cells (reviewed in Bloom and Endow, 1995; Hoyt, 1994).

The Kar3 class of kinesin-related spindle motors are structurally and mechanistically distinct from the *bimC*-like motors. *S. cerevisiae* kinesin-related Kar3p is a minus end-directed motor (Endow *et al.*, 1994; Middleton and Carbon, 1994) with its motor domain at the carboxyl end of the peptide (Meluh and Rose, 1990), whereas the *bimC*-like motors have their motor

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Table 1. Genotypes and plasmids used in this study. All strains are haploid.

Yeast strain	Genotype
WSY32 (wild-type)	*a ade2-101 his3-Δ200 leu2-3 ura3-52*
WSY33 (wild-type)	*α lys2-801 his3-Δ200 leu2-3 ura3-52*
WSY311	*α ade2-101 his3-Δ200 leu2-3 ura3-52 cin8-3 kip1::HIS3*
WSY54	*α lys2-801 his3-Δ200 leu2-3 ura3-52 cin8-3 kip1::HIS3*
WSY165	*a ade2-101 his3-Δ200 leu2-3 ura3-52 cin8-3 kip1::HIS3*
WSY166	*α lys2-801 his3-Δ200 leu2-3 ura3-52 cin8::URA3*
MAY2268	*α lys2-801 his3-Δ200 leu2-3 ura3-52 kar3-102::LEU2*
MAY2371	*α ade2-101 lys2-801 his3-Δ200 leu2-3 ura3-52 kar3-102::LEU2 cin8-3 kip1::HIS3*
MAY2056	*a ade2-101 lys2-801 his3-Δ200 leu2-3 ura3-52 cin8::LEU2*
WSY333	*α lys2-801 his3-Δ200 leu2-3 ura3-52 cin8-1 kip1::HIS3*
WSY55	*α ade2-101 his3-Δ200 leu2-3 ura3-52 kip1::HIS3*
WSY53	*a lys2-801 his3-Δ200 leu2-3 ura3-52 kip1::HIS3*
WSY36	*a lys2-801 ade2-101 leu2-3 ura3-52 cin8-3*
WSY37	*α ade2-101 his3-Δ200 leu2-3 ura3-52 cin8-3*
Plasmids	Relevant plasmid genes
pMR1682	*GAL1:KAR3 URA3 (CEN)*
pBM258	*GAL1:GAL10 URA3 (CEN)*
pMA1214	*GAL1:CIN8 HIS3 (CEN)*
pMR794	*KAR3 URA3 (2 μm)*
pMR1006	*KAR3-1 URA3 (2 μm)*
pYEp24	*URA3 (2 μm)*
pYCp50	*URA3 (CEN)*

domains at the amino end of the peptide chain. There is a growing body of evidence suggesting that Kar3p-like and *bimC*-like motors may act antagonistically in the spindle. Deletion of *KAR3* (indicated by the symbol *kar3-Δ*) markedly but not completely inhibits the inward collapse of the preanaphase spindle observed when cells lose the activity of both the Cin8p and Kip1p motors, suggesting that Kar3p may function to pull together the spindle poles (Saunders and Hoyt, 1992). In a related observation, overexpression of a *KAR3*-like motor in *Aspergillus nidulans*, *klpA*, can block spindle assembly, and deletion of *klpA* can partially rescue the temperature-sensitive phenotype of mutations in the *A. nidulans bimC* motor (O'Connell *et al.*, 1993). In *Schizosaccharomyces pombe*, overexpression of the *KAR3*-like motor PKL1 converts the microtubules structures to a V-shaped or star-shaped array similar to loss-of-function of the *bimC*-like motor *cut7* (Pidoux *et al.*, 1996). These results suggest the existence of two separate types of motive forces acting on the spindle, an outwardly directed motor functioning to separate the spindle poles and an inwardly directed force acting to draw together the spindle poles.

However, it is currently unclear whether these phenotypes represent a truly antagonistic relationship of motors within the spindle. For example, loss of *KAR3* has been suggested to activate a cell cycle checkpoint arrest (Roof *et al.*, 1991), perhaps at a stage where spindles in *cin8 kip1* mutants are less sensitive to collapse. Also, loss of *KAR3* has been reported to produce a higher number of inviable cells (Meluh and Rose, 1990). Perhaps the inviable cells are those that were observed to be resistant to spindle collapse. The existence of a spindle assembly checkpoint, responsive to a variety of cellular defects (reviewed in Wells, 1996) suggests that the observed spindle assembly arrest phenotypes could be due to indirect causes other than excess inwardly directed force, such as altered microtubule dynamics, or a failure to attach chromosomes.

To investigate more directly the relationship between the activities of the Kar3p and Cin8p motors within the spindle, we examined the consequence of overexpression of the genes on spindle structure in wild-type cells arrested with intact spindles. Overexpression of *KAR3* was observed to result in a shrinking of the spindle length, whereas overexpression of *CIN8* initiated spindle elongation with an apparent sliding apart of the two half spindles. These results provide direct evidence that the Cin8p and Kar3p motors act antagonistically in the intact spindle and suggest that their activity must be balanced to achieve normal spindle length. Furthermore, when *KAR3* was overexpressed in *cin8* mutants, anaphase spindle elongation was blocked, suggesting the onset of anaphase spindle elongation may result from a change in the balance of *bimC*-like and *KAR3*-like motors.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains used in these experiments are derivatives of S288C and are listed in Table 1. The *kip1-Δ (kip1::HIS3)*, *cin8-Δ (cin8::URA3)*, *cin8–3* (Hoyt *et al.*, 1992), *kar3-Δ (kar3–102::LEU2)*, and *KAR3–1* alleles (Polaina and Conde, 1982; Meluh and Rose, 1990) have been

described previously. Rich (YPD) and synthetic media were as described (Sherman *et al.*, 1983) with glucose, raffinose, or galactose added to 2%.

Microscopic Analysis of Cells, Imaging, and Spindle Measurements

Antitubulin immunofluorescence was performed on formaldehyde-fixed cells using monoclonal antibodies YOL 1/34 (Serotec, Kidington, United Kingdom) and CY3-conjugated secondary antibodies (Jackson Immunoresearch; West Grove, PA) as described (Pringle *et al.*, 1991; Hoyt *et al.*, 1992). Cells were also stained with the DNA-specific fluorescent dye 4,6-diamidino-2-phenylindole (DAPI, Sigma; St. Louis, MO) to identify the position of the nucleus. Cells were examined with an Olympus B60 epifluorescence microscope using a 100× objective and digital images were captured with an Argus 20 CCD camera and image processor. To increase the percentage of cells in the correct focal plane, all photographic images of antitubulin staining are composites made by combining relevant portions of selected captured images taken from a single sample using Adobe Photoshop software. Spindles were chosen based on clarity and uniform plane of focus throughout the spindle, and care was taken to pick a representative sample. The images were processed using the Photoshop program to make a uniform background. Spindle lengths of Figures 2 and 4 were made from captured antitubulin fluorescent images using the Argus 20 image processor cursor-based measuring function. Distances were measured from the approximate outside edge of the spindle poles. In many but not all cases, the DAPI staining of chromatin was examined to confirm that the cells were not in anaphase. Calibration of the measurement software program was performed by use of an engraved slide (Olympus).

Cell Cycle Arrests and Inhibitors

Hydroxyurea arrest was performed by treating cells with 0.1 M hydroxyurea (Aldrich, Milwaukee, WI) in pH 5.8 YPD, or medium selective for the plasmid, at 26°C for 4 to 5 h. Typically, 65–80% of the cells arrested with large buds and a single nucleus, as determined by fixing the cells with 70% ethanol and staining with DAPI as described (Hoyt *et al.*, 1992). Large-budded cells were defined as those with a bud greater than approximately one-half the diameter of the mother. α-factor arrest was achieved by treating cells with pheromone (Bachem, Torrance, CA) at 6 μg/ml in selective medium at pH 4.0 at 30°C for 3 h. Typically, more than 70% of the cells arrested without buds, as determined by differential interference contrast microscopy. Release from either inhibitor was achieved by resuspending the centrifuged cells in the same medium without inhibitor.

Overexpression Studies

Expression plasmids are listed in Table 1. The *KAR3* gene under the control of the *GAL1* promoter on a *CEN* plasmid (pMR1682) and *KAR3* under the control of its own promoter on a 2-μm plasmid (pMR798) were generously provided by P. Meluh, L. Satterwhite, and M. Rose. pBM258, which contains the *GAL1* and *GAL10* promoters, but not *KAR3* or *CIN8*, was used as a negative control.

Induction of the *GAL* promoter was achieved by growing cells in liquid or on solid medium for about 12 to 15 h with raffinose as the added sugar to derepress the *GAL* promoter. Cells grown in liquid were then resuspended at a lower dilution (typically 1:5) in the same medium with galactose or glucose substituted for raffinose for the indicated times. Cells grown on plates were serially diluted in water and plated on galactose- or glucose-containing medium for 2 to 3 days at 26°C and above and for 5 to 7 days at 22°C. Transformed cells were grown using medium without histidine or uracil to select for retention of the plasmid.

The significance of the changes in spindle length with time of overexpression of the motors was determined by calculating the slope of the line of spindle length versus time with the McCurveFit 1.0.8 program. The slope for p1214 (*CIN8*) was +0.102 with a SD of ± 0.013; for p1682 (*KAR3*) the slope was −0.04 with a SD of ± 0.0036. The p value was computed as the slope divided by the SD of the slope. For both motors the probability that the difference of the slope from zero was due to chance was less than 0.1%.

RESULTS

Effect of Overexpression of KAR3 and CIN8 on Viability

To further investigate the relationship of the *KAR3*-like and *bimC*-like spindle motors on cell viability, the consequence of high copy and *GAL*-driven overexpression of the motors was examined. If these motors act antagonistically, then overexpression would be predicted to be more severe in cells which are defective for the opposing motor.

A high-copy number 2-μm plasmid containing the *KAR3* gene under the control of its own promoter (2-μm *KAR3*) was introduced into wild-type and *cin8 kip1* mutant cells, and the transformed cells were examined at different temperatures. Wild-type cells, with normal levels of Cin8p and Kip1p activity, were unaffected by extra copies of the *KAR3* gene at any of the test temperatures (Figure 1A). Cells containing a temperature-sensitive mutation in the *CIN8* gene (*cin8–3*) and a deletion of the *KIP1* gene (*kip1*-Δ) grew well with extra copies of *KAR3* at lower temperatures, when the *cin8–3* mutant gene product is more active, but were slightly more temperature sensitive. These results suggest that in the presence of excess Kar3p, more Cin8p activity was required for viability. Growth rates of *cin8*-Δ cells containing the 2-μm *KAR3* plasmid were reduced at all temperatures (our unpublished results). In contrast, deletion of the *KAR3* gene partially suppressed the temperature-sensitive phenotype of the *cin8–3 kip1*-Δ mutants, as we have reported previously (Saunders and Hoyt, 1992). Another mutant form of *KAR3*, the *KAR3–1* allele (Polaina and Conde, 1982), has a single amino acid codon change in the putative ATPase site of Kar3p, and although it is able to bind microtubules, it is dysfunctional, with a dominant negative effect on karyogamy (Meluh and Rose, 1990). Unlike wild-type *KAR3*, which increased temperature sensitivity, the *KAR3–1* allele in extra copy partially suppressed the temperature sensitivity of the *cin8–3 kip1*-Δ mutants. This plasmid allowed a plating efficiency comparable to *cin8–3 kip1*-Δ *kar3*-Δ cells (Figure 1A).

To allow inducible high-level expression of these motors, a low-copy *CEN* plasmid containing the *CIN8* or *KAR3* gene under the control of the *GAL1* promoter was introduced into wild-type or *cin8* mutant cells. Transformed cells were grown on raffinose to derepress the *GAL* promoter, and serial dilutions were

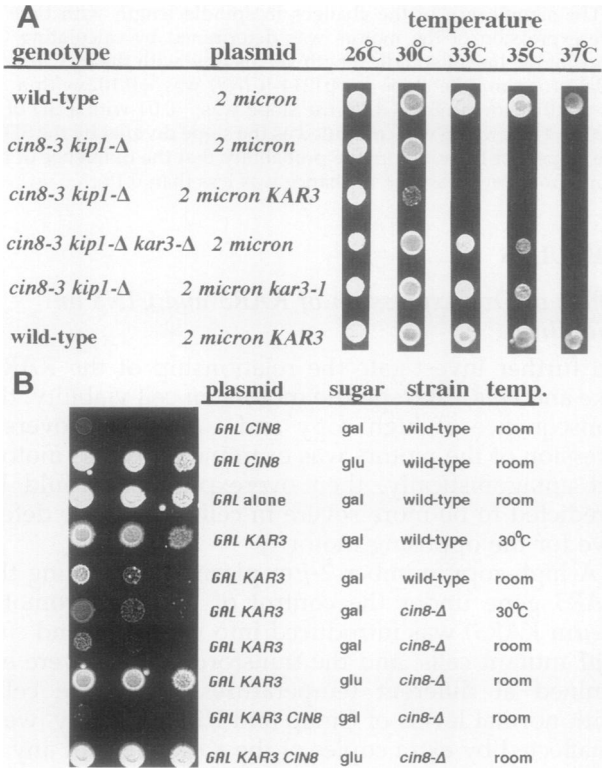

Figure 1. (A) Influence of extra copies of *KAR3* on *cin8 kip1* mutant temperature sensitivity. Cells of the indicated genotypes were transformed with 2-μm-based vectors containing the wild-type *KAR3* gene (pMR798), a *KAR3–1* mutant allele (pMR1006), or with vector alone (pYEp24). Cells were suspended in water, serially diluted, and small aliquots were transferred to selective plates without uracil to retain the plasmid and grown at the indicated temperatures. As shown, extra copies of *KAR3* slightly increased the *cin8 kip1* temperature sensitivity, whereas deletion of *KAR3* or addition of the *KAR3–1* allele partially suppressed the temperature sensitivity phenotype. (B) Influence of *GAL*-induced overexpression of *CIN8* and *KAR3* on cell viability. Cells of the indicated genotypes were transformed with *CEN*-based vectors containing the wild-type *KAR3* (pMR1682) or *CIN8* gene (pMA1214) under control of the *GAL1* promoter or both plasmids together (*GAL KAR3 CIN8*) or a separate plasmid with the *GAL1* and *GAL10* promoters but without *CIN8* or *KAR3* (*GAL* alone; pBM258). Cells were grown overnight on raffinose-containing plates, suspended in water, and serial dilutions of suspended cells were transferred to selective plates containing glucose (to repress the *GAL* promoter) or galactose (to induce expression from the *GAL* promoter) and grown at room temperature (~22°C) for 6 d or at 30°C for 3 d. Overexpression of *CIN8* was toxic at room temperature and at all other tested temperatures. Overexpression of *KAR3* was toxic to wild-type cells only at lower temperatures but to *cin8* null mutants at all temperatures.

plated on galactose to induce overexpression or on glucose to inhibit expression from the *GAL* promoter (see MATERIALS AND METHODS). Overexpression of *CIN8* was found to be lethal to wild-type cells at all temperatures (Figure 1B). (Extra copies of *CIN8*, under control of the endogenous promoter, on a high-copy 2-μm plasmid caused a slight inhibition of growth of wild-type cells; our unpublished results.) Overexpression of *KAR3* had no apparent effect on the viability of wild-type cells at 30°C, but did inhibit growth at room temperature (~22°C; Figure 1B). In contrast, overexpression of *KAR3* was deleterious to *cin8*-Δ cells at all temperatures, although especially so at room temperature. Again, loss of Cin8p apparently made the cells more sensitive to excess Kar3p. Overexpression of both *KAR3* and *CIN8* together in the same cell was lethal (Figure 1B).

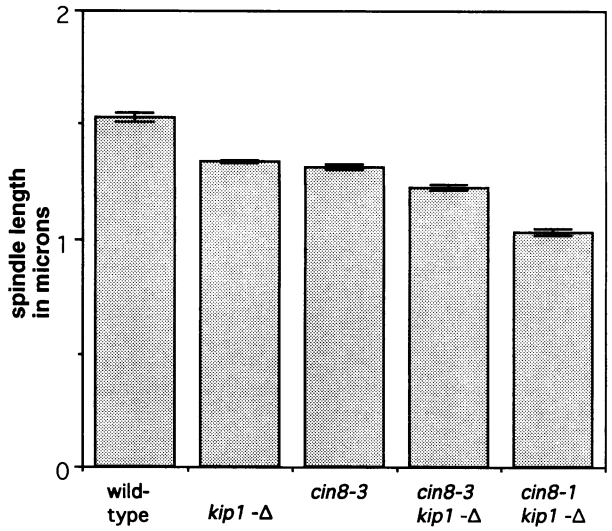

Figure 2. The length of the preanaphase mitotic spindle. Cells with the indicated genotypes were immunostained with antitubulin antibodies, and the length of the spindle was measured by epifluorescence microscopy as described (see MATERIALS AND METHODS). Between 238 and 488 spindles were measured for each genotype. Error bars represent SE of the data.

Changes in Spindle Structure

During the course of these experiments, we noticed that spindles in *cin8 kip1* mutants appeared to be shorter than in wild-type cells. This suggested that we could use spindle length to quantify the effects of changes in *CIN8* and *KAR3* activity. To first confirm the short spindle phenotype of *cin8 kip1* mutants, cells were treated with the DNA synthesis inhibitor hydroxyurea to induce an S-phase arrest (Pringle and Hartwell, 1981). Under these conditions, most cells are able to assemble a spindle but are unable to undergo anaphase until the inhibitor has been removed. Cells of various genotypes were synchronized with hydroxyurea at 26°C, and the length of the spindles was determined after immunofluorescence with antitubulin antibodies (see MATERIALS AND METHODS). Wild-type cells had spindles approximately 1.5-μm long, a finding consistent with earlier electron microscopy studies of *S. cerevisiae* spindles (Peterson and Ris, 1976). Cells with the temperature-sensitive *cin8–3* al-

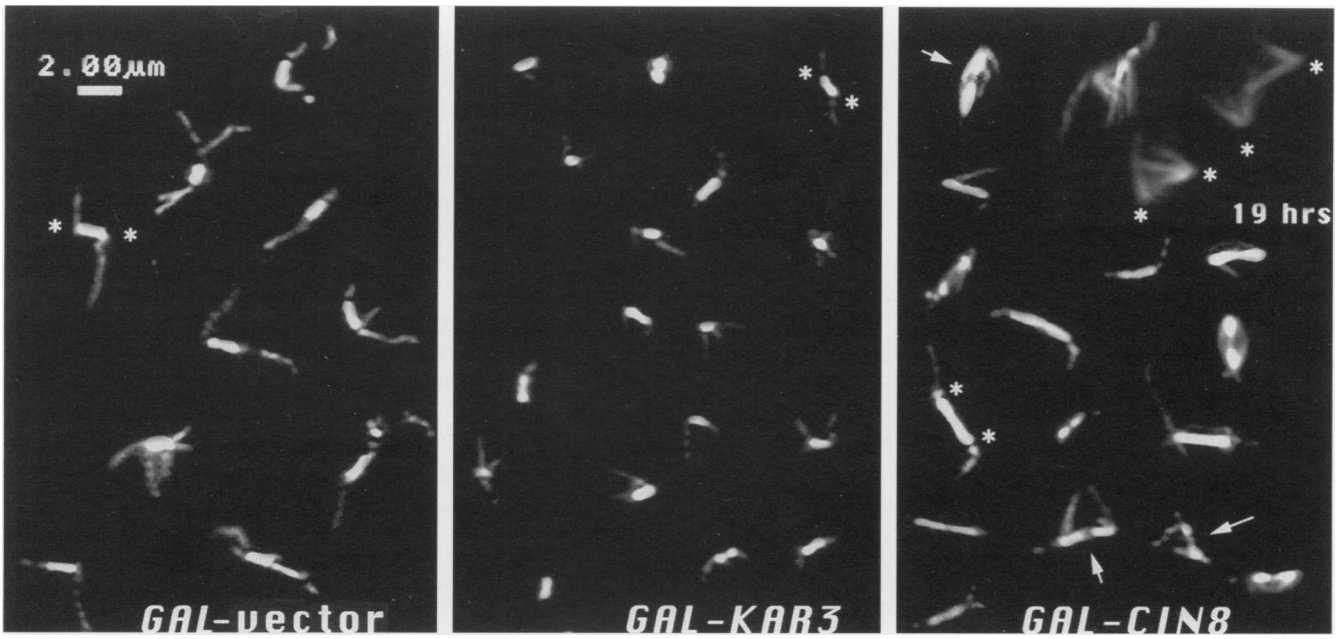

Figure 3. Influence of overexpression of *CIN8* and *KAR3* on spindle structure. Wild-type cells were transformed with plasmids containing *CIN8* or *KAR3* under the control of the *GAL1* promoter or a plasmid containing the *GAL1* and *GAL10* promoters without *KAR3* or *CIN8*. Transformed cells were grown to log phase in liquid media selective for the plasmids, with raffinose as the only added sugar, to derepress the *GAL* promoter. The cells were then resuspended at a reduced density in fresh hydroxyurea-containing medium, with galactose as the sole sugar, for 4 h at 30°C (*CIN8* overexpression and the *GAL* promoter alone) or 26°C (*KAR3* overexpression). Fixed cells were treated for immunofluorescence with antitubulin antibodies (see MATERIALS AND METHODS). Each image shown is a composite of representative cells from a single sample. The inset shows cells that have overexpressed *CIN8* for 19 h. Asterisks, position of representative spindle poles; arrows, regions of apparent decreased microtubule overlap.

lele or the *kip1*-Δ allele had marginally shorter spindles as shown (Figure 2). *cin8–3 kip1*-Δ double mutants contained spindles that were markedly shorter than in wild-type cells. Cells with the *cin8–1 kip1*-Δ mutations are even more temperature sensitive, being inhibited for growth at 30°C compared with 33°C for *cin8–3 kip1*-Δ strains (our unpublished observations). Spindles in *cin8–1 kip1*-Δ mutants were correspondingly shorter than in *cin8–3 kip1*-Δ cells (Figure 2). These results demonstrate that fully functional Cin8p and Kip1p are required for normal mitotic spindle length. Hydroxyurea synchronized cultures of *kar3*-Δ cells had spindles with irregular structure that were difficult to measure for length; however, they did not appear to increase substantially in length. We are currently investigating the nature of the hydroxyurea-arrested spindle defect in *kar3* mutants.

To compare directly the consequence of *KAR3* and *CIN8* activity on spindle length, the effect of overexpression of the motors on spindle structure was determined. Wild-type cells containing the *CIN8* and/or *KAR3* overexpression plasmids (Table 1) were arrested with hydroxyurea. After arrest, cells were resuspended in galactose and hydroxyurea (to maintain the preanaphase arrest while overexpressing the motors) at 26°C for *KAR3* overexpression and 30°C for *CIN8* overexpression and the *GAL* vector alone. The changes in spindle structure over time were determined by examining the cells with antitubulin immunofluorescence. Cells with a plasmid containing the *GAL* promoter without *CIN8* or *KAR3* retained normal spindle structure throughout the hydroxyurea arrest and 4-h galactose induction in hydroxyurea (Figure 3). (When these cells were plated on YPD, before and after galactose induction, the number of viable cells increased slightly, most likely due to some escape from the cell cycle arrest, but showing that the 8-h hydroxyurea treatment did not cause high levels of cell death.) With *CIN8* overexpression, spindles increased in length, typically by about one-quarter to one-third over the course of 4 h (Figures 3 and 4). During this time, gaps of diminished tubulin staining often appeared in the middle of the spindles, suggesting decreased overlap between the two half spindles (Figure 3, arrows). Chromatin separation and the dramatic increase in spindle length (up to 10–12 μm) typical of normal anaphase were not seen. With prolonged overexpression of *CIN8* (19 h), spindles became severely disorganized with little visible association between half spindles (Figure 3, inset). These results suggest that overexpression of the *CIN8* motor

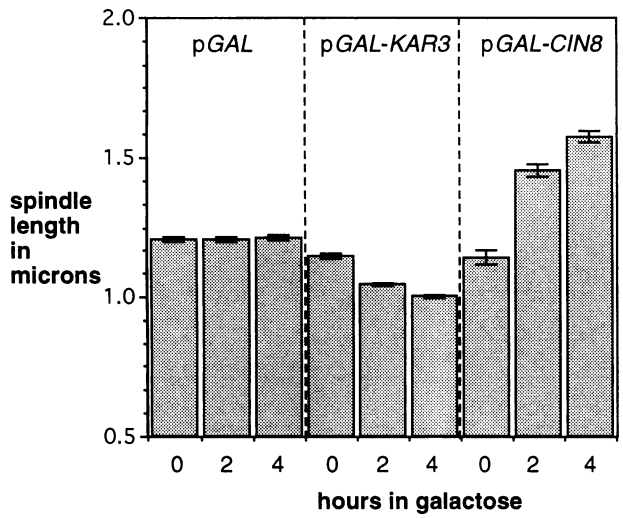

Figure 4. Changes in spindle length with *CIN8* and *KAR3* overexpression. Wild-type cells containing plasmids with the *GAL* promoter alone or the *GAL* promoter controlling transcription of *CIN8* or *KAR3* were arrested with hydroxyurea in selective media plus raffinose (see MATERIALS AND METHODS). Arrested cultures were resuspended in galactose and hydroxyurea for 0, 2, and 4 h, and the lengths of the spindles were determined by fixation, immunofluorescence with antitubulin antibodies, and tracing captured video images (see MATERIALS AND METHODS). As shown, overexpression of *KAR3* caused a slight reduction in spindle length, whereas overexpression of *CIN8* caused an increase in spindle length. The experiments were done at 30°C for *CIN8* overexpression and *GAL* promoter alone or at 26°C for *KAR3* overexpression. The values given represent the average of four different experiments with 200–700 total spindles measured for each time point. Spindles longer than 2.5 μm were not counted to avoid including cells that had escaped the hydroxyurea arrest and entered anaphase. Apparent spindles shorter than 0.5 μm were not counted to avoid including nonspindle microtubule arrays. Error bars represent the SE of the data.

forced spindles to elongate prematurely, apparently causing half spindles to slide apart.

Overexpression of the *KAR3* motor had the opposite effect on spindle structure (Figures 3 and 4). Spindles became progressively smaller. Our impression was that the spindles diminished in size rather than were compressed, as there was no obvious increase in thickness of the spindle. Although the decrease in average spindle length from *KAR3* overexpression was small, the results are significant with p values <0.001 (see MATERIALS AND METHODS). A more dramatic change in the distribution of the different categories of spindle length was also seen. At 0 min of *KAR3* overexpression, 19% (134/698) of the spindles were 1.4 μm or longer, whereas after 4 h only 4.6% (18/395) were 1.4 μm or longer. (Values for the *GAL* vector alone are, respectively, 27% before and 34% after induction.) At 0 min, 24% of the spindles were <1.0 μm, whereas at 4 h 49% were <1.0 μm. (Values for the *GAL* vector alone are 18% before and 15% after induction). The *GAL* vector alone did not cause a significant change in spindle length at 26°C.

Overexpression of *KAR3* did not destroy the spindle structure, as with *CIN8* overexpression, or cause noticeable spindle collapse as described for the *cin8 kip1* mutants (Saunders and Hoyt, 1992), even with prolonged overexpression (19 h). Although the amount of change in spindle length with overexpression of either motor was fairly small, the direction of change was clearly different for *KAR3* and *CIN8*. The effect of overexpression of *KAR3* and *CIN8* together in the same cell was indistinguishable from overexpression of *CIN8* alone (our unpublished results).

Cell Cycle Arrest from KAR3 Overexpression

Although overexpression of *KAR3* in wild-type cells diminished cell growth at lower temperatures, we did not observe a uniform cell cycle arrest phenotype (our unpublished results). However, when Cin8p and Kip1p function was compromised, overexpression of *KAR3* blocked entry into anaphase. *cin8–3 kip1-Δ* cells containing P*GAL-KAR3* or the P*GAL* vector alone were synchronized by the addition of the mating pheromone α-factor at a fully permissive temperature for growth, 22°C. Under pheromone influence, cells reversibly arrest at START of the cell cycle as unbudded cells with a monopolar microtubule array. The cells were then released from α-factor arrest by resuspension in fresh medium containing galactose at either 22°C or a semipermissive temperature of 30°C. At different times cells were removed, fixed with ethanol, and the percentage that had completed mitosis was determined by counting the number of large-budded cells with one (preanaphase or early anaphase) or two (late anaphase or postanaphase) chromatin masses. Large-budded cells were defined as those that had daughters larger than approximately 50% the size of the mother. Most of the *cin8–3 kip1-Δ* cells were able to complete mitosis at 22°C, as shown by the appearance of large-budded cells with two well-divided nuclei (Figure 5B). At 30°C, most of the *cin8–3 kip1-Δ* cells were also able to complete mitosis, but not if *KAR3* was overexpressed (Figure 5D). With *KAR3* overexpression, these cells arrested with large buds and a single undivided nucleus at the neck (Figure 5). Sixty percent of the *cin8–3 kip1-Δ* P*GAL-KAR3* cells arrested with an intact preanaphase spindle under these conditions, as determined by indirect immunofluorescence with antitubulin antibodies. These observations demonstrate that overexpression of *KAR3* can block chromosome separation and spindle elongation if the Cin8p and Kip1p motors are compromised.

DISCUSSION

Antagonistic Spindle Motors

The cytological and genetic data presented here strongly support the existence of antagonistic motors acting on the mitotic spindle. Previous observations by us and others have demonstrated that loss of a Kar3p-like C-terminal motor can partially correct the temperature sensitivity from loss of a *bimC*-like N-terminal motor (Saunders and Hoyt, 1992; O'Connell *et al.*, 1993; Pidoux *et al.*, 1996). We demonstrated here that overexpression of the Kar3p motor can increase the temperature sensitivity caused by mutations in the *bimC*-like motors *CIN8* and *KIP1*. Thus, we can either exaggerate or diminish the *cin8 kip1* temperature sensitivity by increasing or decreasing, respectively, the expression of *KAR3*. This reciprocal genetic relationship is highly suggestive of an antagonistic role for these motors in the cell.

To confirm that the Kar3p and Cin8p/Kip1p motors do in fact act in an antagonistic manner on the spindle, as opposed to influencing growth by some other means, we examined the consequence of changing motor activity on a single, measurable spindle parameter, spindle length. A partial loss of Cin8p and Kip1p activity reduced spindle length, consistent with their proposed outwardly directed force-producing role. Loss of *KAR3* left spindles with abnormal microtubule arrays whose lengths often could not be accurately measured. This abnormal spindle structure is a result of an excess of cytoplasmic microtubules (Saunders *et al.*, 1997). However, by increasing the expression of *KAR3* and *CIN8*, we were able to see a reciprocal change in spindle length.

Overexpression of *CIN8* caused a premature and inappropriate spindle elongation. As the spindles increased in length, it appeared that the half spindles were sliding apart. Surprisingly, overexpression of *CIN8* was able to stimulate spindle elongation in cells blocked for DNA replication, suggesting that duplicated DNA, or an anaphase-specific modification of the motors, is not required for the start of spindle elongation. Rather, it would appear that spindle elongation is dependent on the level of motor activity and can begin at an earlier point in the cell cycle by increasing expression of Cin8p.

The spindle elongation phenotype resembled that observed in purified spindle preparations from diatoms in response to addition of ATP (Masuda and Cande, 1987). In both experimental systems, the half spindles appeared to separate from each other and lose microtubule overlap. However, there are some important differences in the observations from these two systems. In the cell-free diatom system, addition of tubulin led to an increase in microtubule and spindle length similar to the changes seen during anaphase (Masuda and Cande, 1987). In the in vivo

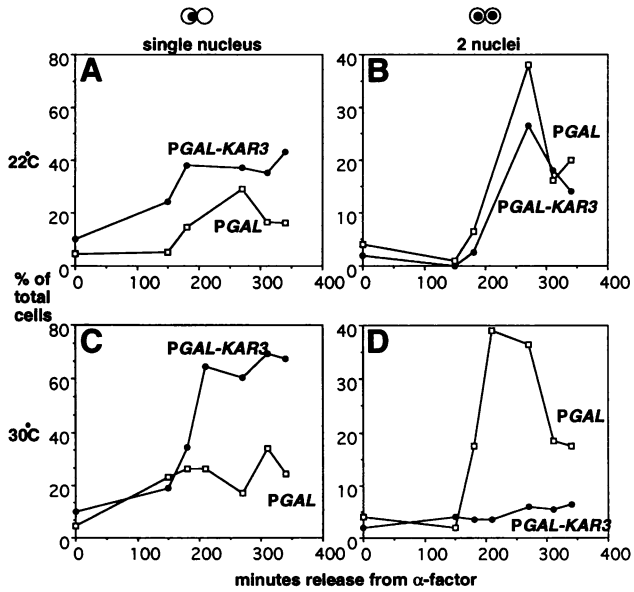

Figure 5. Mitotic arrest from *KAR3* overexpression. *cin8–3 kip1-Δ* cells containing the *GAL-KAR3* overexpression plasmid (p1682; closed circles) or a *GAL* vector alone (pBM258; open squares) were synchronized with α-factor and released in fresh media at 22°C or 30°C. At the indicated times, samples were removed, fixed with ethanol, and stained with DAPI. The cells were examined by epifluorescence microscopy, and the percentage of cells with a large bud (>50% the size of the mother) and one or two chromatin masses over time was determined. Overexpression of *KAR3* was found to block anaphase chromatin separation at 30°C, when Cin8–3p is less active. This is shown by the accumulation of large-budded cells with a single nucleus and the low numbers of large-budded cells with two nuclei at 30°C. Three hundred cells were counted per time point. The release from α-factor at 22°C was performed once; the release at 30°C was done three times with similar results.

conditions used here, although soluble tubulin is present, it fails to polymerize into the gap formed between the half spindles as they slide apart. This observation suggests that in the arrested cells there is some type of inhibition of the additional microtubule polymerization normally seen in anaphase with spindle elongation. The nature of this putative inhibitory mechanism is unknown.

Overexpression of *KAR3* had the opposite effect on spindle structure, causing a shortening of the spindle. This is the first direct demonstration of an opposing action on the intact spindle of *KAR3*-like and *bimC*-like motors. These results suggest that spindle morphology and spindle length are a consequence of the relative activities of both inwardly and outwardly directed spindle motors. Most likely a balance of these two types of motors determines the length of the preanaphase spindle.

This antagonism between Cin8p and Kar3p probably does not result from competition for the same spindle microtubule-binding sites. Kar3–1p from the mutant *KAR3–1* allele can bind to microtubules (Me-

luh and Rose, 1990), but strikingly, extra copies of *KAR3–1* actually decrease the temperature-sensitive phenotype of *cin8–3 kip1-Δ* mutants. Therefore, it is unlikely that the inhibitory effect of wild-type Kar3p is simply due to competitive binding with Cin8p/Kip1p for microtubules. The mechanism by which Kar3–1p suppresses the *cin8 kip1* temperature sensitivity is unknown, but it is interesting that the level of suppression is very similar to deletion of *KAR3*. This suggests that the defective Kar3–1p may be competing with native Kar3p, perhaps for a common binding site or essential cofactor, making the cells effectively null for *KAR3* function. Alternatively, the ATP-binding site mutation in *KAR3–1* may produce a rigor-like cross-linking of the spindle microtubules, preventing the spindle collapse associated with loss of Cin8p and Kip1p activity. Although the results from the *KAR3–1* allele suggest that the effect of overexpression of *KAR3* is not due to direct competition with Cin8p for binding sites on the microtubules, competition for other as yet unidentified shared cofactors cannot be ruled out.

Why Inwardly Directed Motors?

Cin8p and Kip1p have been suggested to cross-link antiparallel microtubules and use those microtubules to push apart the spindle poles during spindle assembly and elongation (Hoyt *et al.*, 1992; Saunders *et al.*, 1995). We propose that Kar3p acts antagonistically to the activity of Cin8p and Kip1p by providing an inwardly directed force acting to pull together the spindle poles.

The need for outwardly directed spindle pole separating motors seems obvious. But it is not immediately clear why the cell would need motors acting to draw together the spindle poles. One possibility is that Kar3p may act as an inhibitory mechanism to delay spindle elongation until the appropriate time in anaphase. In *S. cerevisiae*, the major anaphase B motors Cin8p and Kip1p are needed to separate the spindle poles prior to the onset of anaphase (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders and Hoyt, 1992). Therefore, some other mechanism may initiate spindle elongation at anaphase other than the activation of anaphase B motors. It has generally been regarded that chromatid separation plays a major if not the key role in initiating spindle elongation. In this model, a bipolar chromosome attachment would act as a tether to hold the spindle poles together. However, recently it has been shown that spindle elongation is observed, with the normal timing and range of motion, in grasshopper meiotic spindles with all of the chromosomes removed (Zhang and Nicklas, 1996). Here, we observed the initiation of spindle elongation from *CIN8* overexpression in the presence of the DNA synthesis inhibitor hydroxyurea. These observations imply that although chromatid separation is likely to be an important antecedent, it may not be the primary signal for spindle elongation.

We show that overexpression of *KAR3* can block spindle elongation and chromosome segregation in *cin8 kip1* mutants. This result suggests that Kar3p can act as an anaphase inhibitor. When *Fusarium* mitotic spindles were severed with a laser microbeam, the rate of spindle pole separation was seen to increase threefold, also suggesting the presence of an inhibitor within the spindle (Aist and Berns, 1981; Aist *et al.*, 1993). If Kar3p-like motors can block or slow the rate of spindle elongation, regulation of these motors may play an important role in the initiation of elongation. One possibility is that the Kar3p motors are inhibited or eliminated at or near the onset of anaphase. Consistent with this observation we have reported previously that the spindle collapse in *cin8 kip1* mutants, thought to be Kar3p driven, is no longer observed once anaphase has begun (Saunders and Hoyt, 1992). We have also observed that the epitope-tagged Kar3p was no longer detected at the spindle poles by late anaphase (Saunders *et al.*, 1997). Most likely the onset of anaphase spindle elongation is triggered by a change in the preanaphase balance of inwardly directed and outwardly directed motor activity in favor of spindle elongation.

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REFERENCES

Aist, J.R., and Berns, M.W. (1981). Mechanics of chromosome separation during mitosis in *Fusarium* (fungi imperfecti): new evidence from ultrastructural and laser microbeam experiments. J. Cell Biol. *91*, 446–458.

Aist, J.R., Liang, H., and Berns, M.W.. (1993). Astral and spindle forces in PtK2 cells during anaphase B: a laser microbeam study. J. Cell Sci. *104*, 1207–1216.

Bloom, G.S., and Endow, S.A. (1994). Kinesin. In: Protein Profile, vol. 1, ed. E. Shaterline, London: Academic Press.

Endow, S., Kang, S., Satterwhite, L., Rose, M., Skeen, V., and Salmon, E. (1994). Yeast Kar3 is a minus-end microtubule motor protein that destabilizes microtubules preferentially at the minus ends. EMBO J. *13*, 2708–2713.

Enos, A.P., and Morris, N.R. (1990). Mutations of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans*. Cell *60*, 1019–1027.

Hoyt, M.A. (1994). Cellular roles of kinesin and related proteins. Curr. Opin. Cell Biol. *6*, 63–68.

Hoyt, M.A., He, L., Loo, K.K., and Saunders, W.S. (1992). Two *Saccharomyces cerevisiae* kinesin-related gene-products required for mitotic spindle assembly. J. Cell Biol. *118*, 109–120.

Masuda, H., and Cande, W.Z. (1987). The role of tubulin polymerization during spindle elongation. Cell *49*, 193–202.

Meluh, P.B., and Rose, M.D. (1990). *KAR3*, a kinesin-related gene required for yeast nuclear fusion. Cell *60*, 1029–1041.

Middleton, K., and Carbon, J. (1994). *Kar3*-encoded kinesin is a minus-end-directed motor that functions with centromere binding proteins (CBF3) on an in vitro yeast kinetochore. Proc. Natl. Acad. Sci. USA *91*, 7212–7216.

O'Connell, M.J., Meluh, P.B., Rose, M.D., and Morris, N.R. (1993). Suppression of the *bimC4* mitotic spindle defect by deletion of *klpA*, a gene encoding a *KAR3*-related kinesin-like protein in Aspergillus nidulans. J. Cell Biol. *120*, 153–162.

Peterson, J.B., and Ris, H. (1976). Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. J. Cell Sci. *22*, 219–242.

Pidoux, A.L., LeDizet, M., and Cande, W.Z. (1996). Fission yeast pkl1 is a kinesin-related protein involved in mitotic spindle function. Mol. Biol. Cell *7*, 1639–1655.

Polaina, J., and Conde, J. (1982). Genes involved in the control of nuclear fusion during the sexual cycle of *Saccharomyces cerevisiae*. Mol. Gen. Genet. *186*, 253–258.

Pringle, J.R., Adams, A.E.M., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yeast. Methods Enzymol. *194*, 565–602.

Pringle, J.R., and Hartwell, L.H. (1981). The *Saccharomyces cerevisiae* cell cycle. In: The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance, ed. J.N. Strathern, E.W. Jones, and J.R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 97–142.

Roof, D.M., Meluh, P.B., and Rose, M.D. (1991). Multiple kinesin-related proteins in yeast mitosis. Cold Spring Harbor Symp. Quant. Biol. *56*, 693–703.

Roof, D.M., Meluh, P.B., and Rose, M.D. (1992). Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. *118*, 95–108.

Saunders, W.S., and Hoyt, M.A. (1992). Kinesin-related proteins required for structural integrity of the mitotic spindle. Cell *70*, 451–458.

Saunders, W.S., Koshland, D., Eshel, D., Gibbons, I.R., and Hoyt, M.A. (1995). *Saccharomyces cerevisiae* kinesin- and dynein-related proteins required for anaphase chromosome segregation. J. Cell Biol. *128*, 617–624.

Saunders, W., Hornack, D., Lengyel, V., and Deng, C. (1997). The *Saccharomyces cerevisiae* kinesin-related motor Kar3p acts at preanaphase spindle poles to limit the number and length of cytoplasmic microtubules. J. Cell Biol. *137*, 417–431.

Sherman, F., Fink, G.R., and Hicks, J.B. (1983). Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Yeh, E., Skibbens, R., Cheng, J., Salmon, E., and Bloom, K. (1995). Spindle dynamics and cell cycle regulation of dynein in the budding yeast *Saccharomyces cerevisiae*. J. Cell Biol. *130*, 687–700.

Zhang, D., and Nicklas, R.B. (1996). "Anaphase" and cytokinesis in the absence of chromosomes. Nature *382*, 466–468.