Two *Saccharomyces cerevisiae* Kinesin-related Gene Products Required for Mitotic Spindle Assembly

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**Abstract.** Two *Saccharomyces cerevisiae* genes, \textit{CIN8} and \textit{KIP1} (a.k.a. \textit{CIN9}), were identified by their requirement for normal chromosome segregation. Both genes encode polypeptides related to the heavy chain of kinesin. Cin8p was found to be required for pole separation during mitotic spindle assembly at 37°C, although overproduced Kiplp could substitute. At lower temperatures, the activity of at least one of these proteins was required for cell viability, indicating that they perform an essential but redundant function. Cin8p was observed to be a component of the mitotic spindle, colocalizing with the microtubules that lie between the poles. Taken together, these findings suggest that these proteins interact with spindle microtubules to produce an outwardly directed force acting upon the poles.

**Materials and Methods**

**Yeast Strains and Media**

The yeast strains used in these experiments are derivatives of S288C and are listed in Table I. The original cin8-l, -2, and -3 strains were derived from the collection of strains elevated for mitotic loss of a supernumerary chromosome III (Hoyt et al., 1990). Allelism was demonstrated by linkage analysis of related epitopes to the spindle (Sawin et al., 1992) for linesin-related proteins have been suggested by the localization of related epitopes to the spindle (Sawin et al., 1992) and by genetic observations (see Discussion).

An expected phenotypic consequence of mitotic spindle malfunction is a decrease in the fidelity of chromosome transmission. We have previously reported the identification of *Saccharomyces cerevisiae* mutants that lose a supernumerary chromosome III at elevated rates during mitotic growth (Hoyt et al., 1990). Among this collection of mutants was recognized a subset with defective microtubule structure. New mutant alleles of the two \(\alpha\)-tubulin-encoding loci were identified, as well as mutant alleles of the \textit{CIN1}, \textit{CIN2}, and \textit{CIN4} genes (chromosome instability), required for normal microtubule stability. These findings demonstrated the utility of this chromosome loss method for the identification of gene products that participate in spindle action.

In this paper, we report the genetic identification of the \textit{CIN8} gene by its requirement for normal chromosome transmission fidelity. The \textit{CIN9} gene was identified by its ability to suppress a \textit{cin8} conditional-lethal phenotype. Both genes encode polypeptides related to the heavy chain of kinesin. Analysis of \textit{CIN9} revealed that it is allelic to \textit{KIP1}, identified in a kinesin homology-based screen and also described in the accompanying paper (Roof et al., 1992). Henceforth, we will use the \textit{KIP1} designation. We demonstrated that the \textit{CIN8} and \textit{KIP1} gene products (Cin8p and Kiplp) perform overlapping and essential roles. The function of at least one of these proteins is required to separate duplicated spindle poles during the assembly of the mitotic spindle. Cin8p was observed to be a component of the mitotic spindle, colocalizing with the microtubules that lie between the poles.
Table I. Yeast Strains and Plasmids

| Strain or plasmid | Relevant genotype |
|-------------------|-------------------|
| MAY589            | a CIN8 KIP1       |
| MAY1558           | a cin8-1          |
| MAY1561           | a cin8-2          |
| MAY1563           | a cin8-3          |
| MAY2061           | a cin8::URA3      |
| MAY2077           | a kip1::HIS3      |
| MAY2123           | α::CYH2/a LEU2::CIN1-SUP11-1::leu2 (chromosome III disome) ade2-101 his3-A200 ura3-52 can1Δ cyh2Δ (pMA1200) |
| MAY2166           | same as MAY2123, but kip1::HIS3 |
| MAY2169           | a cin8-3 kip1::HIS3 |
| MAY2177           | a cin8::URA3      |
| MAY2205           | a cin8::LEU2 kip1::HIS3 ura3-52 (pMA1200) |
| MAY2209           | a/α cin8::LEU2/α cin8::LEU2 kip1::HIS3/kip1::HIS3 (pMA1200) |
| MAY2210           | a/α cin8::LEU2/α cin8::LEU2 kip1::HIS3/kip1::HIS3 (pMA1201) |
| MAY2275           | a cin8::URA3 kip1::HIS3 leu2-3,112 cyh2Δ (pMA1208) |
| MAY2305           | a cin8::URA3 kip1::HIS3 lys2-801 (pMA1212) |
| MAY2551           | same as MAY2123, but cin8::URA3 |
| pMA1112           | CIN8 URA3         |
| pMA1125           | CIN8 URA3 (CEN)   |
| pMA1129           | KIP1 URA3 (CEN)   |
| pMA1200           | CIN8-hemagglutinin tag URA3 (CEN) |
| pMA1201           | CIN8-hemagglutinin tag URA3 (2 μm) |
| pMA1208           | CIN8 CYH2 LEU2 (CEN) |
| pMA1212           | CIN8-D99 LYS2 (CEN) |

DNA Manipulations

A URA3-CEN library of S. cerevisiae DNA fragments (Rose et al., 1987) was transformed into both MAY846 (cin8-6) and MAY851 (cin8-2). Ura" transformants were replica transferred to YPD plates and incubated at 37°C. The plasmids from 12 temperature-resistant clones were extracted and transformed into Escherichia coli for analysis. Restriction enzyme analysis revealed that eleven of the plasmids contained genomic DNA inserts that overlapped. A fragment from this locus was introduced into a URA3-containing yeast integrating vector and the resulting plasmid (pMA1122) was cut within the insert and transformed into a ura3 strain. This resulted in a duplication at this genomic locus that is marked with URA3. The strain with the URA3-marked locus was crossed to ura3 cin8-1, -2, and -3 strains and the resulting diploids were sporulated. Only parental genotypes were recovered; all asci analyzed contained two temperature-resistant, Ura" spores and two temperature-sensitive, Ura" spores. This indicated that the isolated genomic DNA contains the bona fide CIN8 locus. The unique genomic insert was found to contain the cin8-suppressing KIP1 locus (originally designated CIN9).

The regions encoding the CIN8 and KIP1 genes were determined by the subcloning of smaller genomic fragments (Sambrook et al., 1989) and by mutagenesis with a modified bacterial Tn10 transposon (Huisman et al., 1987). DNA sequencing was accomplished by subcloning into M13-based vectors and use of the Sequenase reagent kit (United States Biochemical, Cleveland, OH). Only 600 bp of CIN9 were sequenced as this was sufficient to reveal that this locus is equivalent to KIP1 (Rothstein, 1987). Database searches were performed using the FASTA computer program (Pearson and Lipman, 1988). Pairwise comparisons of amino acid sequence similarity and multiple sequence alignments were performed using the GAP, PILEUP, and PRETTY computer programs (Devereux et al., 1984). Adjacent to CIN8 was found the sequence of the end of PRB1, a gene located at the distal tip of the left arm of chromosome V near the CIN1 locus (Mohle et al., 1987; Hoffmann, 1985; Mortimer et al., 1989). This finding agreed with the observed tight linkage of CIN9 to the CIN1 locus (58 PD: 0 NPD: 3 T) and the coincidence of restriction enzymes fragments when CIN8 and CIN1 DNAs were compared. The order on chromosome V is CEN-PRB1-CIN8-CAN1.

The 12CA5 epitope sequence was attached to the 5'-end of the CIN8 open reading frame by oligonucleotide-directed mutagenesis with the Mutα-Gene reagent kit (Bio-Rad Laboratories, Cambridge, MA). The following oligonucleotide was used:

5'-AAAATTGTTAAGATGTTGACAATTACAGGCCTCCAGACTACCTTGAATATTGCUCCCTTCT-3'

The CIN8-D99 allele is a deletion of 99 amino acids from the motor domain of Cin8p that are replaced with seven amino acids from Kip1p (see Results). The following oligonucleotide was used to create CIN8-D99:

5'-GCAAATTCTCGGATGAATGGAATATTGTTGGTATGCAGACAAATCCCTCATTTCCTTTTC-3'

The final mutagenesis products were confirmed by DNA sequencing. Marked deletions of CIN8 and KIP1 were generated by the one-step gene replacement method (Rothstein, 1983). For cin8::LEU2, the XhoI (site -337; see Fig. 1) to BglII (site 1,180) fragment was replaced with a fragment encoding LEU2. For cin8::URA3, the EcoRI (site 942) to EcoRI (site 2,611) fragment was replaced with a fragment encoding URA3. For kip1::HIS3, the SphI–Clal fragment (see Roof et al., 1992) was replaced with a fragment encoding HIS3. Linear DNA from these constructs was used to replace the wild-type alleles in the appropriate strains.

Test for Double Deletion Viability

Crosses between cin8 and kip1 deletion strains yielded double mutant spores that did not germinate into viable colonies. This could reflect either mitotic inviability of the double deletion or an inability of spores of this
to the influenza hemagglutinin protein, cells were double labeled diluted into a paper-filtered solution of 50 mM KI$\text{_4}$ (pH = 7.0), 150 mM conjugated goat anti-rat IgG (Cappel Laboratories). All antibodies were as in (c) 1:250 dilution of YOL1/34; (d) 1:50 dilution of rhodamine-
et al., 1990). Cells fixed to poly-lysine-coated slides were sequentially in-
the visualization of yeast microtubules, relatively short fixation conditions
SPB, spindle pole body.

**Microscopic Analysis of Cells**

To determine the distribution of cell morphologies, culture samples were fixed in 70% ethanol and stained for DNA with 0.5 µg/ml 4,6-diamidino-2-
phenylindole (DAPI) plus 1 mg/ml p-phenylene diamine. Using differential interference contrast optics, cells were scored as un budded, small budded (bud size roughly $\leq$50% size of mother), or large budded (bud size roughly $>50$% size of mother). The number of nuclei per cell was deter-
mined by observation of chromosomal DNA masses by epifluorescent illumination. Microtubule structures were observed after formaldehyde fixation using the anti-tubulin mAb YOL1/34 (Kilmartin et al., 1982; Bio-
products for Science, Inc., Indianapolis, IN) and a fluorochrome-conju-
gated secondary antibody as previously described (Stearns et al., 1990). Spindle length was measured using a calibrated eyepiece reticule. For quan-
titative analyses, at least 200 cells from each sample were examined.

Cells were prepared for thin-section EM as described (Byers and Goetzl, 1991) with the following modifications: glutaraldehyde fixed cells were treated with 50 µg/ml zymolyase at 24°C until a majority of the cells had become spheroplasts (as assayed by loss of refractility when viewed by phase contrast optics; $\sim$t-2 h). Spheroplasts were treated with uranyl acetate overnight at 4°C and dehydrated with successive ethanol baths of 35, 50, 65, 85, 95, and 100% twice each for 5 min.

To visualize the intracellular location of the Cin8p marked with the epi-
tope tag from the influenza hemagglutinin protein, cells were double labeled for immunofluorescence microscopy with mAbs directed against $\alpha$-tubulin [YOL1/34 from rat] and hemagglutinin [12CA5 from mouse (Wilson et al.,
1989)]. Localization of Cin8p in wild-type yeast cells; (b) 1:200 dilution of FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Coehranville, PA) and a fluorochrome-conju-
gated secondary antibody as previously described (Stearns et al., 1990).

**Results**

**Genetic Identification of CIN8 and KIP1**

Three cin8 mutant strains were identified among the previ-
ously described collection of *S. cerevisiae* mutants that seg-
regate a supernumerary chromosome III with reduced fidelity (Hoyt et al., 1990). In growing cultures, a cin8 deletion allele (cin8Δ; see below) caused cells that have lost a marked chromosome III to appear at a 16-fold higher fre-
quency than wild-type at 26°C (Table II). After incubation at 37°C for 12 h, this difference increased to approximately 90-fold. All three original cin8 mutants were recessively temperature sensitive for viability (at 37°C) and failed to complement with each other for this defect. After a shift to the non-permissive temperature, all three strains exhibited a phenotype characteristic of a block at or in mitosis. Cells were predominantly large budded and contained a single nu-
cleus (see below). The arrested cells contained replicated chromosomes, as determined by flow cytometric analysis of DNA content (data not shown).

The CIN8 locus was cloned by complementation of the temperature-sensitive viability defect. 12 rescuing plasmids were isolated from a library of *S. cerevisiae* genomic frag-
ments constructed in a low-copy centromere-containing yeast vector (Rose et al., 1987). 11 of the plasmids contained overlapping DNA inserts that were demonstrated by a link-
age experiment to contain the bona fide CIN8 locus (see Materials and Methods). The CIN8 locus was determined to reside between the PRB1 and CAN1 genes at the distal tip of the left arm of chromosome V (see Materials and Methods).

The last centromere plasmid contained a locus distinct from CIN8 that could suppress the temperature sensitivity of cin8 mutant strains when present in extra copy. The temperature sensitivity of all three original cin8 alleles, as well as inser-
tion and deletion alleles were suppressed by this plasmid. Limited DNA sequence analysis revealed that the suppressing loci were equivalent to KIP1 (Roof et al., 1992). We have not determined the number of excess KIP1 copies required to suppress the temperature sensitivity of cin8 mutants, how-
ever, centromere plasmids are typically maintained at near one or two copies per haploid genome; higher levels cause deleterious effects (Fletcher and Carbon, 1986).

**cin8 and kip1 mutant alleles were generated by in vitro de-
elution of coding sequence and by insertion of a modified bac-
terial TaqI transposon (see Materials and Methods). When transferred back to the genomic locus, all cin8Δ and inser-
tion alleles produced phenotypes similar to the original alle-
les (elevated chromosome loss, Table II, and temperature-
sensitivity for viability at 37°C). In contrast, kip1 mutant

**Table II. Measured Frequencies of Chromosome Loss**

| Strain | Genotype | Median frequency of chromosome loss (X10)$^6$ |
|--------|----------|---------------------------------------------|
| MAY2123 | CIN8 KIP1 | 1.2                                         |
| MAY2551 | cin8Δ KIP1 | 19                                          |
| MAY2123 | CIN8 KIP1 | 0.93                                        |
| MAY2166 | CIN8 kip1Δ | 0.80                                        |

Mitotic loss of a marked copy of chromosome III from disomic haploid strains was measured (see Materials and Methods for details). The values reported represent the frequency of cells resistant to both cycloheximide and canavanine relative to total cells. Frequencies were determined after three days of growth at 26°C from single cells and then after a shift to 37°C for 12 h. All strains were isogenic except for the indicated genotype. The two comparisons were performed on separate occasions and therefore are reported separately.

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1. **Abbreviations used in this paper:** DAPI, 4,6-diamidino-2-phenylindole; SPB, spindle pole body.
The DNA and predicted polypeptide sequence of CIN8. The DNA sequence is numbered on the left with position 1 equivalent to the 3'-end of the PRB open reading frame. Relevant restriction enzyme cut sites are underlined. These sequence data are available from EMBL under accession number ZZ1859 and GenBank under accession number M90522.
strains displayed no detectable mutant phenotype; measured chromosome loss rates were no higher than wild-type (Table II). Strains were constructed that are deleted for both chromosomal CIN8 and KIP1 loci, but carry CIN8 on a plasmid (see Materials and Methods). At temperatures ranging from 11 to 37°C, these strains were unable to remain viable following loss of the CIN8 plasmid. This demonstrated that a functional allele of either CIN8 or KIP1 is required for viability.

Unlike the cin8 deletions, two of the three original cin8 mutant alleles (cin8-1 and cin8-3) could be combined with kipl-Δ to yield viable strains. These double mutant strains grew well at 26°C, but were inviable at 33°C and above. This indicated that the cin8-1 and cin8-3 gene products retain some activity at lower temperature.

CIN8 and KIP1 Encode Kinesin-related Proteins

The observations that the cin8-Δ kipl-Δ genotype is inviable and that extra KIP1 copies suppress the cin8 temperature-sensitive mitotic block suggest a simple hypothesis. Cin8p and Kiplp may perform a redundant but essential function in mitosis. This hypothesis is supported by the finding that the two genes encode related products. CIN8 encodes a 1,038 amino acid polypeptide (118 kD) containing a region (amino acids 67 through 522) with strong similarity to the heavy chain of the microtubule-based mechanochemical enzyme kinesin (Figs. 1 and 2). The sequence of the kinesin-related KIP1 gene is reported in the accompanying paper (Roof et al., 1992).

Genetic and molecular studies of numerous eukaryotic organisms have identified a superfamily of proteins related to the kinesin heavy chain by primary amino acid sequence (reviewed in Endow, 1991; Goldstein, 1991; Rose, 1991). For kinesin, it has been demonstrated that a 340 amino acid NH2-terminal fragment of the heavy chain is sufficient for generating microtubule-based motile force (Yang et al., 1990; Goldstein, 1991). The region of sequence similarity shared by all members of the kinesin superfamily, including Cin8p and Kiplp, corresponds to this 340 amino acid “motor domain” (Fig. 2). The putative motor domains of Cin8p and Kiplp are more closely related to bimC, cut7, and Eg5 than other members of the kinesin superfamily. In contrast, outside of the motor domains, kinesin superfamily members share little or no sequence similarity to kinesin or to each other. This apparently is also the case for Cin8p and Kiplp; outside of the motor domain, Cin8p and Kiplp show only limited sequence conservation (15% identity for the region prior to the motor and 22% for the “tail” region after the motor, as compared with 56% for the motor domain).

The Cin8p motor domain contains a segment of ~100 amino acids that is not present in other characterized kinesin-related proteins (residues 255–353; Fig. 2). It appears in a region of the motor domain sequence that is poorly conserved between members of the superfamily. The function of this segment is not known but is apparently not essential for Cin8p activity. A low-copy plasmid was constructed (pMA1212) that contained a CIN8 gene in which the DNA encoding these amino acids was removed and replaced with a sequence encoding seven amino acids from the corresponding region of Kiplp (amino acids 234–240 = NNNNNSS; see Fig. 2). A strain was then constructed (MAY2305) that carried this plasmid and additionally was deleted for both chromosomal CIN8 and KIP1 loci. This strain was viable and had no obvious defect in growth rate at temperatures ranging from 11 to 37°C.

Loss of Cin8p and Kiplp Function Prevents Spindle Pole Separation

We examined the requirement for Cin8p and Kiplp function during assembly of the pre-anaphase short spindle. The α-factor mating pheromone arrests cells in the G1 phase of the cell cycle, before spindle pole body (SPB) duplication, and subsequent spindle assembly (Byers, 1981). Cell cultures of various genotypes were synchronized with α-factor at 26°C and released from this block into fresh media at 37°C. Observation of the cultures revealed that most cin8-3, cin8-Δ, and cin8-3 kipl-Δ double mutants had not passed through mitosis. After 2 h at 37°C, most cells had arrested growth with a mononucleate, large-budded morphology (Figs. 3 and 4). When α-factor synchronized cells were released onto agar surfaces at 37°C and observed, they arrested growth as single large-budded cells. This indicated that the block to mitosis occurred in the first cell cycle after release. In contrast, following α-factor synchronization and release to 37°C, wild-type and kipl-Δ cells were able to undergo mitosis; mononucleate large-budded cells did not accumulate in these cultures (Fig. 3). Cells of all genotypes were able to pass through mitosis after release from the α-factor block into media at 26°C. Therefore, Cin8p, but not Kiplp, is required to progress from the α-factor arrest point through mitosis at 37°C.

We examined the morphology of the spindles in temperature-arrested mutant cells by anti-tubulin immunofluorescence microscopy (Fig. 4). Wild-type cells formed short (≤1.5–2 μm) bipolar spindles before mitosis. These appeared as a brightly stained bar, corresponding to the nuclear microtubules extending between the SPBs, with fainter staining cytoplasmic microtubules attached to each end (Fig. 4 A). After α-factor synchronization and subsequent incubation at 37°C, short bipolar spindles were not observed in the cin8 single and cin8 kipl double-mutant cells (Fig. 4, C–H). Typically, elaborate cytoplasmic microtubules extended from a single region in each large-budded cell. In arrested cells, virtually all nuclei appeared to be located adjacent to or in the neck dividing the mother and the bud cell bodies. Nuclear migration to the neck, a microtubule-dependent motility event (Huffaker et al., 1988; Jacobs et al., 1988), was therefore not noticeably affected by the cin8 and kipl mutations.

EM of these cells revealed the nature of the cin8 spindle defect (Fig. 5). When released from the α-factor block into media at 26°C, mutant cells assembled normal-appearing spindles with poles separated to opposite sides of the nucleus (Fig. 5 A). In contrast, when incubated at 37°C, spindle pole separation apparently did not occur in either the cin8 single mutant or cin8 kipl double mutant cells (Fig. 5, B and C). SPBs were found adjacent to each other in a side-by-side orientation. In a wild-type cell cycle, before separation, duplicated SPBs are connected by a “bridge” structure (Byers, 1981). In preparations of cin8-3 kipl-Δ cells, we usually observed the presence of a bridge connecting the duplicated SPBs (Fig. 5 C). This observation further supports the conclusion that spindle poles had not separated.
Cin8p Colocalizes with Nuclear Microtubules

The Cin8p sequence similarity to kinesin and the phenotypes of cin8 mutants suggested that this protein interacts with microtubules. We examined this possibility by tagging Cin8p with an epitope from the influenza hemagglutinin protein that is recognized by a mAb (Wilson et al., 1984; Kolodziej and Young, 1991). An oligonucleotide encoding the nine amino acid epitope was fused to the 3'-end of the CIN8 reading frame (see Materials and Methods). The fusion protein thus formed was fully functional for Cin8p activity. Strains were constructed that produced no wild-type Cin8p and Kip1p but were kept viable by the tagged gene carried on a plasmid. These cells were fixed and stained with two mAbs, one directed against tubulin and the other against the hemagglutinin epitope. The antibodies were from two different species, allowing simultaneous labeling of both epitopes with different fluorophores (see Materials and Methods).

When the tagged Cin8p was expressed from a low-copy centromere-containing plasmid, faint anti-hemagglutinin staining colocalized with the spindle microtubules that lie between the poles (Fig. 6, A–E). This staining was only observed on short spindles, however, it is likely that if an equal amount of antigen was present on long spindles, it would be so dilute as to preclude visualization. In no case were anti-hemagglutinin antibodies observed to decorate cytoplasmic microtubules. When the gene encoding the tagged Cin8p was carried by a high-copy 2 μm-based plasmid, the same pattern was observed, but the intensity of nuclear microtubule staining by anti-hemagglutinin was considerably higher (Fig. 6, F–K). In contrast to the low-copy plasmid experiment, staining of long spindles was also observed, although this was a relatively rare occurrence. Again, staining of cytoplasmic microtubules by anti-hemagglutinin was never observed.

The antibody staining of the epitope-tagged Cin8p was often observed to be more intense in regions of nuclear microtubules closer to the spindle poles. The related fission yeast protein cut7 was also recently reported to be localized to nuclear microtubules and is more concentrated near the poles of spindles (Hagan and Yanagida, 1992). In our observations of doubly labeled cells, the staining by both anti-hemagglutinin and anti-tubulin was reduced for intensity in the middle of spindles (i.e., compare Fig. 6, F and G). Therefore, the asymmetric distribution of Cin8p between the poles probably reflects the distribution of nuclear microtubules.

Discussion

In the experiments reported herein, the requirement for Cin8p and Kip1p for normal mitotic chromosome segregation was demonstrated both genetically and morphologically. Our characterization of mutants revealed that the action of these proteins is required for the assembly of the pre-anaphase mitotic spindle. In the absence of both Cin8p and Kip1p functions, duplicated spindle poles did not separate and cells were inviable. No phenotypic consequence was observed for loss of Kip1p function alone and Cin8p function was required for viability only at 37°C. At this temperature, Kip1p must be capable of performing the Cin8p-requiring step since KIP1 in extra copy could substitute for CIN8. These findings taken together are most simply explained by a model in which Cin8p and Kip1p redundantly perform an essential mitotic function.

Cin8p and Kip1p were found to be members of the superfamilies of proteins related to the microtubule-based mechanochemical enzyme kinesin. Mitotic roles for kinesin-related proteins have been suggested by the localization of related epitopes to the spindle (Sawin et al., 1992) and by numerous genetic observations. Although Drosophila kinesin heavy chain gene mutants appear to be unaffected for mitotic function (Saxton et al., 1991), mutant forms of many kinesin-related genes cause mitotic and/or meiotic defects (Davis, 1969; Sequeira et al., 1989; Endow et al., 1990; McDonald and Goldstein, 1990; Carpenter, 1973; Zhang et al., 1990; Meluh and Rose, 1990). Of particular relevance to the experiments reported here, Aspergillus nidulans bimC mutants and Schizosaccharomyces pombe cut7 mutants are defective for mitotic spindle formation at non-permissive temperatures (Enos and Morris, 1990; Hagan and Yanagida, 1990). Strikingly similar to loss of Cin8p/Kip1p function, both bimC and cut7 mutations appear to block the separation of duplicated spindle poles. Of the previously characterized
antil-tubulin  

DAPI
members of the kinesin-related superfamily, the motor
domains of bimC, cut7, Cin8p, Kiplp, and Eg5 from *Xenopus laevis* (Le Guellec et al., 1991) are most closely related in
primary sequence (Fig. 2). Recently, another member of this
bimC-like family has been identified in Drosophila (L. Gold-
stein, personal communication). It therefore seems possible
that this class of kinesin-related proteins is conserved in se-
quency and function across the different eukaryotic phyla.
From the in vitro motility properties of kinesin (Vale et al.,
1985) and the related ncd protein (McDonald et al., 1990;
Walker et al., 1990), it is reasonable to presume that Cin8p,
Kiplp, and relatives are directly involved in the generation
of the force required to separate spindle poles.

For all members of the kinesin superfamily described to
date, little primary sequence conservation is detectable in
comparisons of regions outside of the motor domain. It has
been suggested that the different tails affixed to the motor do-
main may allow diverse "cargos" to be translocated along
microtubules (Goldstein, 1991). Despite their functional
overlap, Cin8p and Kiplp also share extremely limited se-
quency similarity outside of the motor. Therefore, it can be
concluded that tail domains of different primary sequence
may accomplish a similar or a substituting function. Al-
though our findings suggest functional interchangeability, we
imagine that Cin8p and Kiplp may contribute to pole separa-
tion in somewhat different ways.

The consequences of loss of Cin8p and Kiplp function and
their localization to nuclear microtubules (see Roof et al.,
1992 for Kiplp) suggest their direct involvement in spindle
assembly. In the simplest formulation, Cin8p and Kiplp
cross-link interdigitated nuclear microtubules from each
half spindle and slide them past one another to generate an
outward force acting on the poles. Hypothetically, pole sepa-
ration could be accomplished by a motor acting either be-
 tween the poles or on the cytoplasmic (or astral) microtu-
bules located outside of the poles. The epitope-tagged Cin8p
was observed only on the spindle fibers lying between the
poles, although visually undetectable amounts located on the
cytoplasmic microtubules cannot be ruled out. Therefore,
Cin8p most likely generates the pole-separating force from
within the spindle, not from outside of the poles. Experimen-
tal observations by others have suggested that forces that se-
parate spindle poles are produced by interactions between the

Figure 5. Electron microscopic analysis of mutant cells. Cells were
synchronized with α-factor, released into growth media for ~2 h,
fixed, and examined by thin section EM. (A) A cin8-3 kipl-Δ cell
(MAY2169) released into media at 26°C for 2 h. Note the normal-
appearing short mitotic spindle with spindle poles separated to op-
posite sides of the nucleus; (B) A cin8-Δ cell (MAY2061) released
into media at 37°C; (C) A cin8-3 kipl-Δ cell (MAY2169) treated
as in B. The arrow points to the bridge structure connecting the two
SPBs.

Figure 4. Anti-tubulin immunofluorescence microscopy. Cells were
synchronized with α-factor, released into media at 37°C for ~2 h,
fixed, and examined by immunofluorescence microscopy. A, C, E, and
G show anti-tubulin staining and B, D, F, and H show the same
cells stained with the DNA-specific dye, DAPI. (A and B) wild-type (MAY589); (C and D) cin8-3 (MAY1563); (E and F) cin8-Δ
(MAY2061); (G and H) cin8-3 kipl-Δ (MAY2169). Bar, 10 μm.
anti-parallel midzone microtubules (reviewed in Hogan and Cande, 1990).

For S. cerevisiae, the cytoplasmic microtubules appear to be used primarily for positioning of the dividing nucleus and its associated spindle within the cell (Huffaker et al., 1988; T. Huffaker and D. Koshland, personal communications). At the non-permissive temperature, the nuclei in our mutant cells were able to migrate efficiently to the neck dividing the mother and bud cell bodies. Therefore, consistent with the nuclear location of Cin8p, the motility functions provided by cytoplasmic microtubules apparently were unaffected. It is possible, however, that the action of an as yet unidentified cytoplasmic microtubule-based activity can contribute to pole separation in some phase of mitosis (Aist et al., 1991).

We have demonstrated a requirement for Cin8p/Kiplp function for pole separation during spindle assembly. Although not addressed in these experiments, pole separating forces are also required in subsequent mitotic stages. Indeed, we have found that the action of Cin8p or Kiplp is required following the spindle assembly step to oppose a force that draws separated poles back together (W. S. Saunders and M. A. Hoyt, manuscript in preparation). It is possible that these putative motors may function throughout mitosis whenever an outwardly directed force acting upon the spindle poles is required.

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Figure 6. Cin8p colocalizes with nuclear microtubules. Cells deleted for wild-type CIN8 and KIP1, but carrying the hemagglutinin epitope-tagged CIN8 on plasmids were labeled with anti-tubulin (A, C, F, and I), anti-hemagglutinin (B, D, G, and J) and DAPI (E, H, and K) as described in Materials and Methods. The cells depicted in A, B, and C-F (MAX2209) carried the tagged CIN8 gene on a centromere-containing plasmid, while the cells depicted in F-H and I-K (MAX2210) carried the gene on a 2 µm plasmid. Exposure times were optimized for the best image and are not equal. Bar, 10 µm.
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