Kinesin-related Proteins Required for Assembly of the Mitotic Spindle

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Abstract. We identified two new Saccharomyces cerevisiae kinesin-related genes, KIP1 and KIP2, using polymerase chain reaction primers corresponding to highly conserved regions of the kinesin motor domain. Both KIP proteins are expressed in vivo, but deletion mutations conferred no phenotype. Moreover, kip1 kip2 double mutants and a triple mutant with kinesin-related kar3 had no synthetic phenotype. Using a genetic screen for mutations that make KIP1 essential, we identified another gene, KSL2, which proved to be another kinesin-related gene, CIN8. KIP1 and CIN8 are functionally redundant: double mutants arrested in mitosis whereas the single mutants did not. The microtubule organizing centers of arrested cells were duplicated but unseparated, indicating that KIP1 or CIN8 is required for mitotic spindle assembly. Consistent with this role, KIP1 protein was found to colocalize with the mitotic spindle.

The accurate segregation of chromosomes during mitosis is accomplished by a combination of multiple microtubule-based movements (for review see McIntosh and Pfarr, 1991; Sawin and Scholey, 1991). These include migration of the microtubule organizing centers to form a bipolar spindle, chromosome congression to the metaphase plate, chromosome movement along the pole-to-kinetochore microtubules (anaphase A) and spindle elongation due to sliding of the pole-to-pole microtubules (anaphase B).

Although a number of mechanisms have been proposed for the generation of forces that induce movement, it is likely that at least some of the movements are mediated by mechanochemical "motor proteins." One prototype motor protein, kinesin, was identified in squid axons as a protein capable of directing movement of vesicles and organelles toward the plus ends of microtubules in an ATP-dependent fashion (Vale et al., 1985). Moreover, the motor domain has been found at either the NH2 or COOH terminus. In addition to kinesin, motility has been demonstrated for Drosophila ncd (McDonald et al., 1990; Walker et al., 1990). Although it is expected that most of the genes encode motor proteins, it is possible that some of the proteins have nonmotile functions (Roof et al., 1992).

Members of the kinesin superfamily participate in a wide variety of microtubule-mediated processes. For example, the KAR3 protein of Saccharomyces cerevisiae functions in at least two distinct movements (Meluh and Rose, 1990). KAR3 is required for nuclear fusion, where it probably serves to move the two nuclei of a zygote together via the cytoplasmic microtubules. kar3 mutants also accumulate inviable cells that are blocked in mitosis. Their morphology suggests that KAR3 participates in spindle elongation. Mutations in kinesin-related genes bimC of Aspergillus nidulans (Enos and Morris, 1990) and cut7 of Schizosaccharomyces pombe (Hagan and Yanagida, 1990) prevent the separation of the spindle pole bodies necessary to form a bipolar mitotic spindle. Mutations in the Drosophila genes ncd and nod principally affect meiotic chromosome transmission, but ncd may also play a role in mitosis (Davis, 1969; Carpenter, 1973; Sequeira et al., 1989; Zhang and Hawley, 1990). Drosophila khc mutations affect neuromuscular functions (Saxton et al., 1991), as does the Caenorhabditis elegans unc-104 mutation (Hall and Hedgecock, 1991). These are only a subset of the kinesin-related genes, since in Drosophila the existence of 11 kinesin-related genes has been verified (Steward et al., 1991), but as many as 35 may exist (Endow and Hatsumi, 1991).

Since several different motors and a variety of movements occur within a single cell, some degree of spatial and temporal specificity of each motor exist. Specificity determinants have been postulated to lie in the tail sequences

1. Abbreviations used in this paper: 5FOA, 5-fluoorotic acid; khc, kinesin heavy chain; PCR, polymerase chain reaction.
which are unique to each protein, but sequences in the motor domain could confer specificity as well. However, the multiplicity of kinesin-related proteins within a single organism raises the possibility of functional overlap wherein several different motor proteins power a single movement. For example, KAR3 cannot be the sole force-generating protein that participates in mitotic spindle elongation, because kar3 null mutants are viable although slow growing (Meluh and Rose, 1990). Presumably other force-generating proteins also serve to power this movement. Such functional redundancy might explain the absence of kinesin-related genes among the collection of cell division cycle (CDC) genes in yeast. It is not yet known to what extent individual motors are restricted to a single movement, and to what extent several different motors can overlap in function.

We report here the existence of multiple kinesin-related proteins in S. cerevisiae. KIP1 and KIP2 were identified by their homology with kch and KAR3. Using a genetic screen for mutations that make KIP1 essential for viability, we identified another gene, KSL2. KSL2 was found to be allelic with CIN8, another kinesin-related gene (Hoyt et al., 1992). KIP1 and CIN8 exhibit functional redundancy in spite of the dissimilarity of their tail sequences. We have used conditional double mutants to show that these proteins mediate migration of the duplicated spindle pole bodies at the onset of mitosis. Immunofluorescent localization of KIP1 protein shows that it is present on early mitotic spindles.

### Materials and Methods

#### Strains and Microbial Techniques

The yeast strains used are listed in Table II. Media and genetic techniques were as described in Rose et al. (1990). Yeast transformations were by the method of Hoffman and Winston (1987).

#### Polymerase Chain Reaction

Polymerase chain reaction amplification of kinesin-related genes was done using degenerate primers that correspond to conserved regions of KAR3, bimC, and kch. Primer 1 encodes the peptide IFAYGQT (5'ggagacatgacgaccagcgtctcgccaggtttgggaattc-3') and is 131,000-fold degenerate. Primer 4 is the antisense to amino acids 211 using a BglII site within the yeast DNA insert. The 182-bp insert DNA for the k/p/-6::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase and digestion with Sinai. The 182-bp insert DNA for the k/p/-6::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase and digestion with Sinai. The 182-bp insert DNA for the k/p/-6::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase and digestion with Sinai. The 182-bp insert DNA for the k/p/-6::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase and digestion with Sinai.

#### Isolation of the KIP1 and KIP2 Genes

A YCP50 plasmid-based yeast genomic library was screened by colony hybridization (Davis et al., 1980) using gel purified cloned polymerase chain reaction (PCR) amplified DNA as probes. Candidate plasmids were analyzed by restriction digest and Southern blotting to identify plasmids with the hybridizing sequences near the middle of the yeast DNA insert. pMR1690, pMR1691, and pMR1692 carry KIP1, and pMR1695 and pMR1697 carry KIP2.

#### Plasmid Constructions and DNA Sequencing

For sequencing KIP1, the 5,734-bp BgII fragment from pMR1692 was cloned in both orientations into pKS (+) to make pMR1722 and pMR1723. As this fragment was found not to include the KIP1 NH2 terminus, a partial overlapping 1,708-bp EcoRl-PstI fragment from pMR1690 was cloned into pKS (+) and pKS(−) to make pMR1703 and pMR1702. For sequencing KIP2, the 3,819-bp EcoRI-BglII fragment from pMR1697 was cloned into pKS (+) and pKS(−) to make pMR1705 and pMR1706. To sequence beyond the BglII site which is located 20 bp from the COOH terminus of KIP2, the 671-bp PstI-EcoRI fragment from pMR1695 was cloned into pKS (+) to make pMR2362. Nested deletions of the above plasmids were made using exonuclease III and nuclease S1 (Hoheisel and Pohl, 1986), and single-stranded DNA was sequenced using Sequense (United States Biochemical, Cleveland, OH). Both strands of the KIP coding regions were sequenced.

KIP1 was subcloned into the yeast shuttle vectors constructed by Sikorski and Hieter (1989). The 5,093-bp NheI fragment from the KIP1 plasmid pMR1691 was cloned into the SpeI site of pRS316 (a CEN plasmid marked with URA3) to pRS315 (CEN LEU2) and pRS306 (YIp URA3) to make pMR1895, pMR1893, and pMR1891, respectively.

For insertional mutagenesis and immunological detection of KIP1, DNA fragments which encode three copies of the hemagglutinin epitope were introduced into four restriction sites of pMR1893, to create in-frame insertions. The kipl-5::HA insertion was made at KIP1 amino acid residue 211 using a BglII restriction site, the kipl-6::HA insertion was made at residue 754 using MluI, the kipl-7::HA insertion was made at residue 54 using AluI and the kipl-7::HA was made at residue 1,022 using Accl. Since the kipl-5, kipl-6, and kipl-7::HA sites were not unique, pMR1893 DNA was partially digested with the restriction enzyme and singly cut plasmid DNA was purified by agarose gel electrophoresis. The linear DNA fragments were treated with large fragment of DNA polymerase I and 4 dNTPs to generate blunt ends. The DNA fragments encoding the epitope were isolated from plasmid GTEPI, obtained from B. Pucher (Cold Spring Harbor Laboratory, NY). This plasmid contains a Nod fragment that encodes three copies of the sequence YPYDVPDYA, in pBluescript II SK (+). The 143-bp insert DNA for the kipl-4::HA and kipl-5::HA alleles was made by digesting GTEPI with BstXI, removing the 3' extension with T4 DNA polymerase and digestion with Smal. The 182-bp insert DNA for the kipl-6::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase, digestion with CiaI, and treatment with alkaline phosphatase to yield a large fragment to generate the second blunt end. The 151-bp insert DNA for the kipl-7::HA allele was made by digesting GTEPI with SacI, removing the 3' extension with T4 DNA polymerase and digestion with Smal. The insert fragments were purified by agarose gel electrophoresis and ligated with the appropriate linearized KIP1 plasmid DNA. Plasmids containing insertions in the desired restriction sites and in the correct orientation were identified by restriction digestion and gel electrophoresis of plasmid DNA isolated from E. coli transformants.

DNA and predicted protein sequence analysis was performed using the Sequence Analysis Software Package by Genetics Computer, Inc.

#### Construction of kipl1 and kipl2 Deletion Strains

The null allele kiplA1::HIS3 is an internal deletion of KIP1 corresponding to amino acids 106 to 1,046, replaced by HIS3. pMR1892 was digested with BclI to remove three BclI fragments internal to KIP1, and a fragment containing HIS3 was inserted to create pMR1921. The null allele kipla2::TRPI is an internal deletion of KIP2 corresponding to amino acids 94 to 667, replaced by TRPI. pMR1775 was partially digested with XhoI, this fragment that resulted from cutting only at XhoI sites within KIP2 was gel purified, and a fragment containing TRPI was inserted to create pMR1791. The kipla2::URA3 allele in pMR1790 was made using a fragment encoding UR43 instead of the TRPI fragment. The resulting kipl deletion strains are MS2333 and MS2334, and the kipl2 deletion strains are MS2309 and MS2354.
The *kip* deletion mutations were inserted into the wild-type genomic *KIP* genes using the one-step gene replacement technique of Rothstein (1983). pMR1921 was digested with EcoRI plus XhoI, and pMR1790 and pMR1791 were digested with XhoI plus SphI before transformation. The structure of each deletion mutation was confirmed using Southern blots of genomic yeast DNA prepared from the deletion mutants.

**Mapping of KIP1 and KIP2**

*KIP1* was assigned to chromosome II by hybridization of a *KIP1* probe to electrophoretically separated yeast chromosomes. The *KIP1* gene was localized to a position near *ils1* on the left arm of chromosome II by hybridization of the *KIP1* probe to dot blots of mapped yeast DNA fragments kindly provided by L. Riles and M. Olsen (Washington University School of Medicine, St. Louis, MO). Linkage to *ils1* was confirmed using meiotic crosses. The *kip1*::URA3 strain MS2305 was crossed to the *ils1* strain IL785 and sporulated. 42 tetrads were parental ditype and one was tetatype, indicating that *KIP1* is 1 cM from *ils1* (calculated using the formula of Perkins, 1949).

*KIP2* was assigned to the left arm of chromosome XVI by hybridization of a *KIP2* probe to electrophoretically separated yeast chromosomes and chromosome fragments. Sequence of the *KIP1* region revealed that the likely *KIP2* initiation codon is located 326 bp downstream of the termination codon of the *PEP4* gene (see Fig. 2).

**Immunological Techniques**

Total yeast protein for Western blots was extracted from exponentially growing cells by the method of Ohashi et al. (1982), resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membrane was incubated with mAb 12CA5 ascites fluid, which recognizes an epitope (termed HA) from the influenza hemagglutinin protein (Wilson et al., 1984). Alternatively, a polyclonal anti-kinesin peptide antibody was used (Sawin et al., 1992). After incubation with secondary antibody conjugated to HRP (Amersham Corp., Arlington Heights, IL), protein was detected using the ECL detection kit (Amersham Corp.).

Immunolocalization was performed by the methods of Adams et al. (1984) and Kilmartin and Adams (1984) as modified in Rose and Fink (1987). For detection of HA epitope-tagged proteins with the 12CA5 antibody, cells were fixed with formaldehyde for 30 min at 23°C. Tubulin staining was done with rabbit anti-tubulin antibody RAPI/24, a gift of F. Solomon (Massachusetts Institute of Technology, Cambridge, MA). Goat anti-rabbit antibody conjugated to fluorescein isothiocyanate, or conjugated to rhodamine, and goat anti-mouse antibody conjugated to fluorescein isothiocyanate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNA was stained using the fluorescent dye 4,6-diamino-2-phenyindole (DAPI) (Boehringer Mannheim Biochemicals).

**Electron Microscopy**

Cells were pregrown at 23°C and shifted to 37°C for 3 h in YMI medium (Hartwell, 1967), then fixed and embedded for sectioning by the method of Byers and Goetsch (1991). Cells with a large bud and at least one spindle pole body were identified, and adjacent sections were scored for the presence or absence of a second spindle pole body.

**Chemical Mutagenesis**

Strains MS2335 and MS2336 were mutagenized for the synthetic lethal mutant screen with ethyl methanesulfonate as described in Rose et al. (1990), plated for single colonies on YPD medium, and replica printed to 5-fluorouracil (5FOA) (Boeke et al., 1987) medium (all at 30°C) to identify 5FOA sensitive derivatives. Temperature sensitive *kip1* mutations were identified using the plasmid shuffle technique (Boeke et al., 1987). For this purpose, plasmid DNA was mutagenized with hydroxylamine as described in Rose and Fink (1987) and used to direct transform yeast. Putative *kip1* (ts) plasmids were recovered from yeast by transformation into E. coli, and the BamHI Xbal fragment containing *kip1* was cloned into the URA3-marked Ylp vector pRS406 (Silkorski and Hieter, 1989). The resulting plasmids were ligated with BglII or MluI and used to replace the chromosomal copy of *KIP1* with the *kip1(ts) allele by plasmid integration and excision (Scherer and Davis, 1979).

**Results**

**Identification of the Kinesin-related Genes KIP1 and KIP2**

Members of the kinesin superfamily possess several regions of sequence conservation within their putative motor domains. To identify new kinesin-related genes, we amplified yeast genomic DNA by PCR using degenerate oligonucleotide primers corresponding to three regions highly conserved between *KAR3*, *khc*, and *bimC*. One of the primer sites includes part of an ATP binding/hydrolisis consensus sequence (Walker et al., 1982), while the other two primer sites are in regions implicated in microtubule binding (Yang et al., 1989). The primer sites flank additional conserved regions, whose presence in an amplified DNA fragment signifies identification of a kinesin-related gene.

Two genes with homology to the motor domain of kinesin were identified by cloning and sequencing the DNA fragments generated by PCR amplification. We have designated the genes *KIP1* and *KIP2*, for kinesin-related protein. To obtain the complete genes, the cloned amplified DNA fragments were used as hybridization probes to screen a yeast genomic DNA library. DNA fragments carrying *KIP1* or *KIP2* were identified by hybridization, subcloned, and their DNA sequences were determined. The *KIP1* open reading frame would encode a peptide of 1,111 amino acids (Fig. 1), while the *KIP2* open reading frame would encode a peptide of 706 amino acids (Fig. 2). The chromosomal location of *KIP1*, as determined by hybridization of a *KIP1* probe to a mapped yeast DNA library and by meiotic crosses, is on the left arm of chromosome II near *ILS1*. *KIP2* was located by DNA sequencing of a known adjacent gene and resides on the left arm of chromosome XVI next to *PEP4* (see Materials and Methods). Neither gene has been previously described.

The NH2-terminal region of the predicted *KIP1* protein and a central region of the predicted *KIP2* protein show extensive sequence similarity to the force generating domain of *khc* (Fig. 3 A). The *KIP1* and *Drosophila melanogaster* *khc* sequences show 42% amino acid identity over the central 335 residues of this domain (defined in Rose, 1991), and the *KIP2* sequence shows 38% identity. The sequence conservation includes the consensus sequence GX,GKT proposed to contribute to ATP binding and hydrolysis (Walker et al., 1982), as well as other sequences present in the domain of kinesin thought to be required for microtubule binding (Yang et al., 1989). *KIP1* shows particularly high sequence similarity with the putative motor domains of *bimC*, *cut7*, and *Xenopus Eg5* (Le Guellec et al., 1991) (65, 54, and 52% identity, respectively, over 335 amino acids), suggesting that these proteins comprise a subgroup of highly conserved kinesin-related genes.

In contrast to the motor domain, the COOH-terminal 690 residues of *KIP1* show little or no sequence similarity with the nonmotor regions of any of the kinesin-related proteins, nor has substantial sequence similarity been found with sequences in the current GenBank database using the TFASTA search program. Like many members of the kinesin superfamily, the nonmotor region of *KIP1* encodes several regions containing heptad repeats of hydrophobic and charged amino acids (Fig. 3 B). Such regions are associated with the forma-
Figure 1. Nucleotide and predicted protein sequence of KIP1. The GXGKT sequence proposed to contribute to ATP binding and hydrolys
sis (Walker et al., 1982) and the BclI restriction sites that form the boundary of the kip1Δ deletion mutation are underlined. KIP1 encodes a predicted protein of 1,111 amino acids and 126 kD. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z11962.

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Kinesin-related Proteins Required for Mitosis

The kipl and kip2 deletion mutations result in haploid as well as diploid yeast that show normal growth, meiosis, and karyogamy. The deletion mutations of both kipl and kip2 individually play no obvious role in mitotic functions of yeast: mitosis, meiosis, and karyogamy. However, deletion of both kipl and kip2 null mutants were then examined for defects in the three known microtubule-dependent functions of yeast: mitosis, meiosis, and karyogamy. The deletion mutations of both genes could be recovered in haploid and diploid yeast and mutants showed wild-type growth rates on rich medium (Conde and Fink, 1976). Sporulation of the resulting x kip1 x kip2 generated diploids at the wild-type frequency when measured using a qualitative limited mating assay (Walker et al., 1982) and the XhoI restriction sites that form the boundary of the kip2Δ deletion mutation are underlined.

The GX.GKT sequence proposed to contribute to ATP binding and hydrolysis (Walker et al., 1982) and the XhoI restriction sites that form the boundary of the kip2 deletion mutation are underlined. KIP2 encodes a predicted protein of 706 amino acids and 78 kD. The COOH-terminal predicted protein sequence of KIP2 is shown. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z11963.

The predicted protein sequence of KIP2. The GX.GKT sequence proposed to contribute to ATP binding and hydrolysis (Walker et al., 1982) and the XhoI restriction sites that form the boundary of the kip2Δ deletion mutation are underlined. KIP2 encodes a predicted protein of 706 amino acids and 78 kD. The COOH-terminal predicted protein sequence of KIP2 is shown. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z11963.
Figure 3. Sequence comparison of motor domains and probabilities of coiled-coil formation by KIP1 and KIP2. (A) Sequence comparison of the motor domains of *S. cerevisiae* KIP1, KIP2, KAR3, and CIN8, *A. nidulans* bimC and *D. melanogaster* khc. Amino acids identical to KIP1 are indicated as white characters on black, and gaps introduced to facilitate alignment are indicated with periods. The sites of PCR primers used to identify KIP1 and KIP2 are marked with heavy lines (D), and the GX4GKT sequence for ATP binding is marked with asterisks (*). The spacing of the conserved regions in the predicted KIP2 sequence differs from other lineins family members due to an insertion of 28 amino acids at residue 402, and CIN8 differs due to an 84-amino acid insertion at residue 253. The sequences were aligned using the Pileup computer program (Genetics Computer, Inc.). The bimC sequence (amino acids 69-488) is from Enos and Morris (1990), CIN8 (63-591) is from Hoyt et al. (1992), khc (1-399) is from Yang et al. (1989), and KAR3 (375-729) is from Meluh and Rose (1990). (B) The probability that each residue of KIP1 and KIP2 is part of a coiled-coil structure was calculated using the algorithm of Lupas et al. (1991), using a window size of 28 amino acids.
homozygous null diploid strains yielded tetrads with >90% spore viability.

The lack of an obvious phenotype conferred by the kipl and kip2 mutations was surprising because both KIP1 and KIP2 are expressed in wild-type strains during vegetative growth (data shown below). One possible explanation is that these kinesin-related proteins overlap in their essential function, so that a mutation in a single gene has little effect. Functional redundancy was tested with the KIP1, KIP2, and KAR3 genes by constructing double and triple mutant strains. All of the double and triple mutants were viable. The kipl kip2 double mutant showed a wild-type growth rate, while all of the mutants containing the kar3 deletion mutation grew at a reduced rate, due to a defect in mitosis as described previously (Meluh and Rose, 1990). The kipl and kip2 mutations did not exacerbate the growth defect of kar3 strains. Several possibilities could explain the lack of an observable phenotype. One is that KIP1, KIP2, and KAR3 are not the sole members of a functionally redundant group. Alternatively the group may not be required for an essential process.

Like the single mutants, the kipl kip2 double mutants mated and formed diploids at wild-type frequencies when crossed either with wild-type or mutant strains. The homozygous diploids could be sporulated to yield tetrads with >90% spore viability. Thus KIP1 and KIP2 are not conjoinedly required for either karyogamy or meiosis. The various kar3 mutants could not be tested because KAR3 is essential for both nuclear fusion and meiosis.

ksl Mutants Make KIP1 Essential for Growth

As suggested above, the lack of a phenotype conferred by the kipl and kip2 deletion mutations can be explained by proposing the existence of yet other force generating protein(s) that can substitute or compensate for the loss of KIP1 or KIP2. In that case, loss of either gene individually might cause no gross defect, but simultaneous loss of both genes would be lethal (synthetic lethality). We therefore designed a genetic screen to identify mutants in which KIP1 is essential for mitotic growth. This genetic screen could potentially identify the genes for force generating proteins which might not have been detected using our PCR primers, as well as other genes required for the activity of an additional motor protein.

The synthetic lethal mutant screen is based upon an assay in which the requirement for a given gene is assessed by determining whether a mutant strain is able to segregate a plasmid bearing that gene as its sole copy (Kranz and Holm, 1990; Bender and Pringle, 1991). Specifically, the parent strains (MS2335 and MS2336) were deleted for the chromosomal KIP1 gene and were also ura3+, but carried a functional KIP1 gene on a centromere-based plasmid marked with URA3 (pMR1895). The parent strains frequently segregate the plasmid during vegetative growth and consequently become ura3+. Ura3+ but not Ura3- strains can grow on medium containing 5FOA (Boeke et al., 1987). Colonies of the parental strains grown on rich medium contain many Ura+ segregants; upon replica plating to 5FOA these clones appear to be drug resistant owing to the large number of Ura+, 5FOA resistant cells. In contrast, putative synthetic lethal mutants cannot remain viable after plasmid segregation; these appear as 5FOA sensitive colonies owing to lack of viable Ura+ cells.

Screening of 22,000 mutagenized cells identified 18 inde-pendent 5FOA+ mutants. These were potential ksl mutants (kip synthetic lethal). All of the mutants were found to be recessive for the Ksl phenotype. The mutations fell into four complementation groups; ksl1 had 14 alleles, ksl2 had two alleles, and two mutations were unique.

To determine whether the 5FOA+ mutants now required a functional KIP1 gene, or were 5FOA+ for reasons unrelated to KIP1 function, we performed a secondary screen. Potential ksl1 mutants were transformed with a second KIP1 plasmid which carried a LEU2 selectable marker instead of URA3 (pMRR1893). In mutants which require KIP1 for viability, the newly introduced KIP1 gene should complement the ksl1- kipl- defect, rendering the original URA3-marked KIP1 plasmid dispensable. This would consequently permit accumulation of ura3+ cells and the strains would again appear to be 5FOA+. Representatives of each ksl complementation group were tested, and it was found that only the two ksl2 alleles fulfilled the criteria of becoming 5FOA+. The remaining mutants remained 5FOA+ and were not studied further.

Meiotic crosses were used to demonstrate the synthetic lethality between kipl and ksl2 independent of the plasmid segregation test. The kiplΔ ksl2 mutant MS2839 was crossed to the kiplΔ1 strain MS2336 and sporulated. Since a KIP1+ plasmid was present, tetrads with three and four viable spores could be recovered because the plasmid KIP1 gene complemented the defect of kiplΔ1 ksl2- spores. In the four spore tetrads, two spores were 5FOA+ and two were 5FOA-, indicating that the Ksl+ phenotype is caused by a mutation at a single locus. The KIP1 plasmid in the above heterozygous diploid strain could be segregated, confirming that the ksl2 mutation is recessive. When the diploid strains without the KIP1+ plasmid were sporulated, two spores in each tetrad were viable and two were inviable, indicating that in the absence of KIP1 function, the ksl2 mutation is lethal. Thus mutants singly defective in KIP1 or KSL2 are viable, but the double mutant is inviable.

KSLS and Kinesin-related CIN8 Are the Same Gene

During the course of this work, the S. cerevisiae CIN8 gene was cloned and identified as another kinesin-related gene (Hoyt et al., 1992). Certain mutations in the CIN8 gene result in an increased rate of chromosome loss and temperature sensitive growth. KIP1 and CIN8 show particularly high sequence identity within their putative motor domains (Fig. 3A, 56% identity), but show no substantial similarity in their COOH-terminal regions. Like KIP1, CIN8 is a member of the bimC/cut7/Eg5 subgroup, although KIP1 is more closely related to bimC and cut7 than it is to CIN8.

Functional redundancy between kipl and cin8 was tested by crossing kiplΔ1::HI53 strain MS2333 to cin8Δ::LEU2 strain MAY2058 and examining the meiotic products. The spores inferred to carry both the kipl and cin8 mutations were inviable, indicating synthetic lethality. When the KIP1 URA3 plasmid pMRR1895 was present, the double mutants were viable but 5FOA+, confirming that KIP1 is essential in a cin8 background.

Complementation and allelism tests were used to determine whether the ksl2 and cin8 mutations define the same gene. As described above, ksl2 kipl and cin8 kipl double mutants are viable when a KIP1 URA3 plasmid is present; these strains appear as 5FOA+ colonies due to the lethality of plasmid loss. For complementation testing, these double mutants...
Table I. Cell Type Distribution of Single and Double Mutants

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>Time at 37°C h</th>
<th>No bud</th>
<th>Small bud</th>
<th>Medium bud</th>
<th>Large bud, 2 nuclei, elongated spindle</th>
<th>Large bud, 1 nucleus, short spindle</th>
<th>Anucleate</th>
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* Bud size was scored by Nomarski microscopy, nuclei were visualized by staining DNA with DAPI, and microtubule organization was examined by indirect immunofluorescent staining of tubulin. Anucleate cells were scored for each time point.

were used to make diploid strains. The cin8Δ kip1Δ1/pKIP1 URA3 strain MY2875 was crossed to the ksl2-1 kiplΔ1/pKIP1 URA3 and ksl2-2 kiplΔ1/pKIP1 URA3 strains MS2839 and MS2868. The diploids were 5FOA³, indicating that the plasmid-based KIP1+ gene remained essential because ksl2 and cin8 did not complement. Sporulation of these diploids yielded no spores that could become 5FOA R, indicating that the ksl2 and cin8 mutations are tightly linked. We have redesignated ksl2-1 to be cin8-101 and ksl2-2 to be cin8-102.

Conditional kipl cin8 Double Mutants Arrest before Spindle Pole Body Migration

The synthetic lethality between the kiplΔ1 and the cin8 mutations indicates that either KIP1 or CIN8 is sufficient to perform a function essential for viability. To determine the nature of the essential function, we generated temperature-sensitive alleles of KIP1 in the cin8-101 background, and examined the phenotype of the double mutant at the non-permissive temperature.

To isolate kipl temperature-sensitive alleles, we mutagenized the wild-type KIP1 gene on plasmid pMR1893, and used the "plasmid shuffle" procedure (Boeke et al., 1987) to identify plasmids that were conditional for their ability to complement the kiplΔ1 cin8-101 defect of strain MS2879. Three temperature-sensitive alleles, kipl-101(ts), kipl-102(ts), and kipl-103(ts) were isolated. The kipl(ts) genes from these plasmids were recloned in an integrating vector and used to replace the wild-type chromosomal KIP1 gene of several yeast strains by plasmid integration and excision. The temperature-sensitive phenotype could be recovered after plasmid excision in the cin8-101 point mutant background (MS2909) as well as in a cin8 deletion background (MAY2059).

The gene replacement procedure allowed dominance or recessivity of the kipl(ts) alleles to be easily determined because integration of the kipl(ts) plasmids created a kiplΔ1/KIP1+ merodiploid. The three merodiploids were temperature resistant in both the cin8-101 and cin8 deletion backgrounds, indicating that the kipl(ts) alleles are recessive.

The kipl-101(ts) cin8-101 double mutant cells were examined by light microscopy after incubation at 37°C to determine whether the mutant arrests at a specific stage of the cell cycle. The kipl-101(ts) cin8-101 strain was pregrown at 23°C, shifted to 37°C for 5–6 h, then fixed and stained. A nearly uniform arrest morphology was observed after incubation at 37°C for 3 h. About 75% of the cells in the arrested culture had a single large bud (Table I). Staining of DNA with the fluorescent dye DAPI showed that the large budded cells contained only a single nucleus often located in or near the bud neck (Fig. 4). In comparison, the nucleus in wild-type large budded cells had already divided and segregated into the mother and daughter cells (data not shown).

Microtubule organization was examined using indirect immunofluorescent staining of tubulin. The microtubules of the arrested cells appeared to emanate from a single pole and did...
not span the width of the nucleus. The vertex of the microtubules at the edge of the nucleus was usually located near the bud neck, and appeared brighter and thicker than the microtubules characteristic of unbudded wild-type cells (Fig. 4). In some cells, two distinct bundles of microtubules converged at the pole, forming a "V" structure. This phenotype is suggestive of a block after spindle pole body duplication but before the formation of the bipolar mitotic spindle.

In addition to the large-budded cell type, the arrested culture contained a significant number of abnormal unbudded anucleate cells. The fraction of anucleate cells increased from 7 to 33% when incubation at 37°C was prolonged to 5 h, suggesting that the anucleate cells were derived from the large budded cells when cytokinesis continued in the absence of nuclear division. This phenotype is similar to that seen with the cut mutants of S. pombe, including mutants defective in a kinesin-related gene, cut7, which is required for bipolar spindle formation (Hagan and Yanagida, 1990).

We also examined the effect of a single kiplΔ1 or cin8-10l mutation on the distribution of cell types. The kiplΔ1 strain cell type distribution was similar to wild type at 23 and 37°C (Table I), suggesting that under these growth conditions cin8 can completely compensate for loss of kipi. In contrast, the cin8-10l single mutant showed an abnormal cell type distribution at 37°C. After 3 h at 37°C, about 50% of the cells had a large bud and a single nucleus, and 3% of the cells were anucleate (Table I). Many of the cells with a large bud and a single nucleus contained microtubules that appeared to emanate from a single pole with a morphology similar to that of the arrested kipl(ts) cin8-10l double mutant. However, the cin8-10l single mutant culture also contained cells with more mature spindles than those seen in the kipl(ts) cin8-10l double mutant. The microtubules of a few of the large-budded cells with a single nucleus appeared to emanate from two poles, forming a short bright-staining bar, indicating that a bipolar spindle had formed. The cin8-10l culture also contained 5–9% cells with a large bud and two separate nuclei (Table I). In these cells the microtubules spanned the distance between the nuclei, a characteristic of normally fully elongated mitotic spindles. The fraction of cin8-10l cells in the 37°C culture with elongated spindles was similar to that seen for the wild-type strain, consistent with the wild-type growth rate exhibited by the cin8-10l mutant at 37°C. The presence of cells with elongated spindles suggests that the cin8-10l mutation causes a pause at the onset of mitosis, or that some cells bypass the abnormal state.

However, the large number of abnormal cells in the cin8-10l culture indicates that kipi does not duplicate all cin8 functions.

Since the kipl(ts) cin8-10l double mutants appeared to be defective in converting the short monopolar spindle into a bipolar spindle, we wanted to determine whether the kipi/ cin8 function is required before or after spindle pole body duplication. Spindle pole bodies were examined by EM of arrested double mutant cells. The diploid double mutant MS2923 was pregrown at 23°C, shifted to 37°C for 3 h, and the cells were prepared for EM. Several serial sections were examined for 11 large-budded cells with at least one spindle pole body. A second spindle pole body was observed in 8 cells (Fig. 5). The two spindle pole bodies were usually located immediately adjacent to each other. These results demonstrated that the spindle pole bodies could be duplicated, but that spindle pole body migration failed to occur, resulting in a monopolar spindle as the terminal structure of the double mutant at the restrictive temperature.

**Both the NH2-terminal and COOH-terminal Domains Are Required for KIP1 Activity**

Since kipi and cin8 exhibit functional redundancy in spite of the dissimilarity of their tail sequences, we investigated whether mutations in the kipi tail affected cin8 function. The effect of mutations in the kipi NH2-terminal and COOH-terminal domains was tested by introducing in frame insertions of DNA fragments at four sites (Fig. 6A) and testing the ability of the altered kipi genes to complement the kiplΔ1 cin8-10l growth defect. To allow immunological identification of the proteins, we inserted DNA fragments encoding three repeats of the hemagglutinin peptide recognized by the mAb 12CA5 (Wilson et al., 1984). The insertions introduced 49–61 codons into kipi, carried on plasmid pMR1893. Complementation was tested using the plasmid shuffle technique after transformation of strain MS2879. The COOH-terminal kipi-7::HA insertion allele complemented the kiplΔ1 cin8-10l defect at 23, 30 and 37°C, while the NH2-terminal kipi-4::HA allele complemented only at 23 and 30°C. In contrast, kipi genes bearing insertions in the motor region (kipi-5::HA) and near the middle of the COOH-terminal domain (kipi-6::HA) failed to complement at any temperature. Similar amounts of full-length Kipi protein were detected for the functional kipi-4::HA allele and the noncomplementing kipi-5::HA and kipi-6::HA alleles by Western blotting of protein from cells grown at 23°C, sug-

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**Figure 5.** Spindle pole body duplication in kipl(ts) cin8-10l arrested cells. Electron micrographs (A–C) each show a section of different MS2923 diploid cells arrested at 37°C for 3 h. Two spindle pole bodies are visible in each cell. Bar, 200 nm.
KIP1: Localization to Mitotic Spindles

The cellular location of KIP1 was examined using indirect immunofluorescent staining of yeast strains carrying the KIP1-4::HA or the KIP1-7::HA epitope-tagged gene. The epitope-tagged gene was carried on a single copy CEN plasmid and was expressed from its own promoter. In these experiments, the epitope tagged gene was the sole source of KIP1 in both a CIN8+ and a cin8-101 background. Medium and large-budded cells frequently showed staining of short bars, which coincided with anti-tubulin staining of short mitotic spindles (Fig. 7). In addition, KIP1 staining in unbudded and small-budded cells was often detected as a dot located at, or near the spindle pole body. Overall, ~25% of the cells showed staining using either the KIP1-4::HA or the KIP1-7::HA epitope-tagged genes. The observed variation in staining intensity may reflect the potential low abundance of KIP1 protein and the sensitivity of the HA epitope to fixation. The same localization pattern was observed in the CIN8+ and cin8-101 genetic backgrounds. These data suggest that KIP1 is a normal participant in spindle pole body migration, and not simply an auxiliary motor induced or relocalized in response to a cin8-101 defect.

Discussion

Our results demonstrate the existence of multiple members of yeast harboring the tagged plasmids (Fig. 6 B). All of the epitope-tagged KIP1 plasmid strains contained a single reacting protein species. The KIP1-4::HA fusion protein had a ~134 kD, which is consistent with the predicted fusion protein molecular mass of 131 kD. The level of KIP1 expression was identical in each genetic background (Fig. 6 B), indicating that KIP1 is not induced in response to a defect caused by the cin8-101 mutation.

We used an antibody raised against the conserved motor region peptide HIPYRESKLT to visualize potential kinesin-related proteins (Sawin et al., 1992). At least six proteins were detected in a wild-type strain by Western blotting (Fig. 6 C). To investigate whether the detected proteins correlate to known genes, total protein was prepared from yeast stains deleted for single kinesin-related genes. A protein of ~121 kD was present in all strains except the kipl deletion strain, indicating that this protein is the likely product of KIP1. As was the case with epitope-tagged KIP1, the level of KIP1 protein detected with the HIPYR antibody was similar in CIN8+ and cin8 deletion strains, confirming that KIP1 expression is unaltered by a cin8-101 defect.

KIP2 protein was also detected using the HIPYRESKLT antibody. An 83-kD protein was present in all strains except the kip2 deletion strain, indicating that KIP2 is expressed during normal growth. No proteins could be positively assigned to the KAR3 and CIN8 genes, as all proteins detected in the wild-type strain were also present in the kar3 and cin8 deletion strains. The HIPYRESKLT antibody may not recognize these proteins since they are not conserved at the site used to raise the antibody. KAR3 differs in two residues (HIPFRNSKLT) and CIN8 differs in one residue (HIPFROLSKLT). The remaining four proteins were ~54, 66, 112, and 190 kD. All four were detected in kipl, kip2, kar3, and cin8 deletion strains, suggesting that additional S. cerevisiae kinesin-related proteins exist.

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Discussion

Our results demonstrate the existence of multiple members
was evident because mutants defective in both of the kinesin superfamily in yeast. Two of the kinesin-related genes, KIP1 and CIN8, were shown by mutant analysis to perform an essential role in mitosis. The essential role was evident because mutants defective in both KIP1 and CIN8 are inviable. However, mutants singly defective in KIP1 or CIN8 are viable, indicating that either wild-type gene can provide the essential function missing in the double mutant. Therefore, KIP1 and CIN8 are functionally redundant. Functional redundancy between KIP1 and CIN8 is supported by the finding that one or two extra copies of the KIP1 gene on a plasmid can suppress the temperature sensitivity caused by certain cin8 point mutations (Hoyt et al., 1992).

The essential function of KIP1 and CIN8 was examined using a double mutant containing a kipl temperature sensi-

tive allele and a cin8 point mutation allele. The double mutant showed a nearly uniform cell division cycle arrest morphology at the nonpermissive temperature. Light microscopy showed that the arrested cells have a single large bud, a single nucleus and a short monopolar spindle. EM revealed that most large-budded cells have duplicated spindle pole bodies. The arrest morphology suggests that the KIP1 and CIN8 proteins mediate spindle pole body separation and migration to form a bipolar spindle. Consistent with this role, immunolocalization of KIP1 protein showed that it is a component of short mitotic spindles. The mitotic spindle localization of KIP1 is similar to the mitotic spindle localization of S. pombe cut7 protein reported recently (Hagan and Yanagida, 1992), although we rarely detected KIP1 protein on elongated spindles.

Particularly high sequence similarity within the putative motor domains defined a subgroup of kinesin-related proteins. Whereas most kinesin related proteins contain between 30 and 45% identity within the motor region, the motor regions of KIP1 and A. nidulans bimC showed 65% identity, KIP1 and S. pombe cut7 showed 54% identity, and KIP1 and Xenopus Eg5 showed 52% identity. CIN8 is also a member of the subgroup; it shows 56% identity with KIP1. Presumably, the high sequence similarity within the bimC/cut7 subgroup reflects a common cellular function for these proteins. Consistent with this suggestion, the bimC and cut7 gene products appear to play similar roles early in mitosis, as mutations in either gene can block separation of the newly duplicated spindle pole bodies (Enos and Morris, 1990; Hagan and Yanagida, 1990).

Redundant Kinesin-related Proteins

Several possible mechanisms could explain how either one of two different motors is sufficient to perform an essential role. One possibility is that only one of the two motors is normally used. A defect in the primary motor might lead to induction or relocalization of a second motor so that it could perform as a substitute. The second "auxiliary" motor could be induced by a mitotic checkpoint arrest mechanism that monitors the progress of mitotic spindle formation similar to that previously described (Hoyt et al., 1991; Li and Murray, 1991). A second possible explanation is that both motors normally act in tandem to power a single movement, but either motor acting alone is sufficient. Finally, it is possible that successful completion of one movement can compensate for the loss of a second movement. For example, incomplete spindle elongation (anaphase B) might be not be lethal when anaphase A proceeds normally, but would be lethal when anaphase A is also defective. The combinatorial use of mitotic motors is probably necessary to achieve high fidelity chromosome transmission.

Contrary to the inductive auxiliary motor hypothesis, KIP1 is expressed at the same level and shows apparently identical localization in both cin8- and CIN8+ strains. The converse of the induction hypothesis is that CIN8 would be induced in response to a KIP1 defect. However, cin8- single mutants have an altered cell type distribution at 37°C, with many cells that are arrested or delayed at an early stage of mitosis. These results suggest that CIN8 is expressed and plays an important role in KIP1+ strains. Therefore, CIN8 does not serve solely as an auxiliary motor that is induced in response to a kipl defect. We expect that insights into the
An important issue is what makes CIN8 and KIP1 partially interchangeable. Presumably there is a feature common to both KIP1 and CIN8 that allows this pair of proteins to be interchanged. Since the kinesin-related proteins all possess interchangeable motor domains with KIP1 and CIN8 are capable of complementing the motor regions. In this regard, it will be of interest to determine whether the other kinesin-related proteins that show particularly high sequence conservation in the motor domains with KIP1 and CIN8 are capable of complementing the kip1 cin8 double mutant defect.

The mitotic defect of mutants defective in CIN8 alone indicates that the KIP1 and CIN8 proteins are not completely interchangeable. No defect was detected in the kip1 single mutant, indicating that CIN8 can completely substitute for KIP1. However, both KIP1 and CIN8 could perform additional roles which are not redundant with each other, but that do overlap with other force generating proteins. If such additional force generating proteins exist, the genes could potentially be identified using the ksl mutant screen. Since the ksl mutant screen for synthetic lethal mutants with KIP1 has yielded only two cin8 alleles, the screen is not exhausted and could potentially identify other force generating proteins that overlap with a different KIP1 function.

Functional overlap among force-generating proteins may be a general phenomenon. The multiple movements of mitosis may be powered by force generating proteins that have various degrees of functional overlap with each other. A second S. cerevisiae kinesin-related protein that is likely to have partial functional overlap with other force-generating proteins of mitosis is KAR3. The morphology of kar3 mutants indicates that this protein participates in anaphase spindle elongation (Meluh and Rose, 1990). The viability of kar3 null mutants suggests a second protein can partially fulfill the mitotic function of KAR3. A third potential example of motor redundancy is KIP2. The KIP2 protein is expressed, but no defect in kip2 null mutants could be detected, raising the possibility that another force-generating protein overlaps KIP2 function. Recently, synthetic lethal mutants for KIP2 have been isolated (D. Roof, D. Loayza, and M. Rose, unpublished results). In addition to genetic arguments for the existence of additional force-generating proteins, the spectrum of redundancy will be gained by high resolution localization studies of KIP1 and CIN8. Of course either KIP1 or CIN8 could be an auxiliary motor that is regulated at the level of activation rather than expression or localization.

Table II. S. cerevisiae Strains Used

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* All strains are derivatives of strain S288C, and all contain the ura3-52 and leu2-3,112 mutations. Strain MS10 is from the lab collection. MS524 is from Meluh and Rose (1990), MAY2058 and MAY2059 were obtained from M. A. Hoyt (Johns Hopkins University, Baltimore, MD), and L785 was obtained from G. Fink (Whitehead Institute, Cambridge, MA). All other strains were constructed for this study. Yeast genes carried on plasmids are indicated in parentheses after the plasmid name. KIP1::HA designates an in frame insertion that encodes three copies of the HA epitope. 

† Derived from MAY2058.
of yeast proteins recognized by the anti-kinesin peptide anti-body suggests that at least four additional kinesin-related proteins exist in \textit{S. cerevisiae}.

We would like to thank Tammy Mandell for technical assistance in generating the \textit{kip} \textit{(ts)} mutants, R. Patel for sectioning cells for EM, A. Hoyt and W. Saunders for providing strains and information before publication, K. Sawin and T. T. 1973. A metiotic mutant defective in distributive disjunction in \textit{Drosophila melanogaster}. Genetics. 73:393–428.


