Loss of Function of *Saccharomyces cerevisiae* Kinesin-Related *CIN8* and *KIP1* Is Suppressed by *KAR3* Motor Domain Mutations

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ABSTRACT

The kinesin-related products of the *CIN8* and *KIP1* genes of *Saccharomyces cerevisiae* redundantly perform an essential function in mitosis. The action of either gene-product is required for an outwardly directed force that acts upon the spindle poles. We have selected mutations that suppress the temperature-sensitivity of a *cin8*-temperature-sensitive *kip1*-Δ strain. The extragenic suppressors analyzed were all found to be alleles of the *KAR3* gene. *KAR3* encodes a distinct kinesin-related protein whose action antagonizes Cin8p/Kip1p function. All seven alleles analyzed were altered within the region of *KAR3* that encodes the putative force-generating (or "motor") domain. These mutations also suppressed the inviability associated with the *cin8*-Δ *kip1*-Δ genotype, a property not shared by a deletion of *KAR3*. Other properties of the suppressing alleles revealed that they were not null for function. Six of the seven were unaffected for the essential karyogamy and meiosis properties of *KAR3* and the seventh was dominant for the suppressing trait. Our findings suggest that despite an antagonistic relationship between Cin8p/Kip1p and Kar3p, aspects of their mitotic roles may be similar.

DURING mitosis, the microtubule-based mitotic spindle undergoes a well defined series of motility events (reviewed in HYAMS and BRINKLEY 1989; INOUE 1981; MCINTOSH and PFARR 1991; NICKLAS 1988). The various spindle movements are complex and probably result from the actions of a number of force-generating mechanisms. A major research objective for the mitosis field is the identification and functional characterization of the force-generating molecules that drive these movements.

The assembly of the bipolar spindle structure from duplicated spindle poles is an early mitotic motility event. In this stage, duplicated poles are separated by the anti-parallel microtubule array that forms between them. Recently, six related gene-products that apparently function in this stage have been identified in diverse eukaryotic species. Conditional *bimC* mutants of *Aspergillus nidulans* and *cut7* mutants of *Schizosaccharomyces pombe* fail at bipolar spindle assembly when incubated at nonpermissive temperatures (ENOS and MORRIS 1990; HAGAN and YANAGIDA 1990). For *Saccharomyces cerevisiae*, it appears that two gene-products overlap in their ability to perform spindle assembly (HOYT *et al.* 1992; ROOF, MELUH and ROSE 1992). Although at the extreme growth temperature of 37° the function of *CIN8* is essential for this process, at lower temperatures either *CIN8* or *KIP1* function alone is sufficient to assemble the spindle. The Eg5 protein was found to be essential for the assembly of spindles in extracts made from *Xenopus laevis* eggs (SAWIN *et al.* 1992). Finally, *Drosophila melanogaster urchin* mutants, defective for the function of the klp2 protein, display centrosomal separation defects (P. WILSON and M. FULLER, unpublished data; M. HECK, unpublished data).

The products of the aforementioned six genes share a region of primary sequence similarity to the force-generating (or "motor") domain of the mechanochemical enzyme kinesin (YANG, LAYMON and GOLDSTEIN 1989; ENOS and MORRIS 1990; HAGAN and YANAGIDA 1990; HOYT *et al.* 1992; LE GUELLEC *et al.* 1991; STEWART *et al.* 1991). Pairwise sequence comparisons revealed that these six are more closely related to each other within the motor domain than to kinesin or other kinesin-related proteins. It therefore seems probable that an essential aspect of spindle assembly is accomplished by this type of kinesin-related protein in diverse eukaryotic cells. From their resemblance to kinesin, it is reasonable to presume that these proteins are directly involved in the production of a pole-separating force.

We previously reported that the action of either *S. cerevisiae* gene-product, Cin8p or Kip1p, is also required following spindle assembly to maintain the integrity of the bipolar structure (SAUNDERS and HOYT 1992). When the activities of both were eliminated, pre-anaphase bipolar spindles rapidly collapsed, with previously separated poles being drawn together. In contrast, anaphase spindles were apparently resistant to collapse. Of particular relevance to this paper

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was the finding that the deletion of the gene encoding another kinesin-related protein, *KAR3*, could partially suppress loss of Cin8p/Kip1p function. A potentially analogous situation has recently been observed in *Aspergillus* (O'CONNELL *et al.* 1993). These authors reported that deletion of the *KAR3*-related *klpA* gene suppressed the temperature-sensitivity of an allele of the *CIN8/KIP1*-related *bimC* gene. These findings suggest that the pre-anaphase mitotic spindle is stabilized by counteracting forces produced by different kinesin-related proteins. One force is acting to push poles apart and is presumably produced by Cin8p and Kip1p in *S. cerevisiae*. This outwardly directed force is countering a force that pulls poles together and to which Kar3p appears to be making a contribution.

In this paper we report the original observations that demonstrated that *KAR3* alleles can suppress loss of Cin8p/Kip1p function. A set of *KAR3* missense alleles selected as suppressors of this mutant phenotype were characterized. The *KAR3* alleles are not null for function and actually suppress loss of Cin8p/Kip1p function better than a complete *KAR3* deletion. The properties of these suppressing alleles of *KAR3* suggest that despite an antagonistic relationship with Cin8p/Kip1p, aspects of their mitotic roles may be similar.

MATERIALS AND METHODS

Yeast strains and media: The yeast strains used in these experiments are derivatives of S288C and are listed in Table 1. The *cin8-3*, *cin8::URA3*, *cin8::LEU2*, *CIN8:URA3* and *kip1::HIS3* alleles are described in HOYT *et al.* (1992). The *kar3-102::LEU2* deletion is described in MELUH and ROSE (1990). Rich (YPD), minimal (SD) and sporulation media were as described (SHERMAN, FINK and HICKS 1983).

Selection of *cin8 kip1* pseudorevertants: MAY2082 was plated for single colonies on YPD medium at 26°. Individual colonies were resuspended in water and plated on YPD at 35°. Fast growing colonies were picked for further analysis. To ensure independence of the suppressing mutations, only a single pseudorevertant was chosen from each original 26° colony.

Recovery and sequencing of the suppressing alleles of *KAR3*: DNAs carrying the suppressing alleles of *KAR3* were obtained by the recombinational repair of a gapped *KAR3*-containing plasmid (ROTHSTEIN 1991). A *Bam*HI-*Cla*I fragment containing the entire *kar3-1* gene was obtained from pMR1011 (a gift from P. MELUH and M. ROSE) and inserted into the *URA3*-centromere vector pRS316 (SIKORSKI and HIETER 1989) to generate pMA1235. Cutting pMA1235 with *Eco*RI plus *Bgl*II removed almost the complete *kar3-1* open reading frame [see MELUH and ROSE (1990) for restriction map]. The DNA fragment containing the entire vector and the up and downstream sequences from *kar3-1* was purified from an agarose gel and transformed into seven yeast strains carrying suppressor mutations. Plasmid DNA was recovered from Ura^+ transformants and used to transform the *Escherichia coli* strain DH5α (SAMBROOK, FRITSCH and MANIATIS 1989). The plasmids obtained were tested for suppressing activity after transformation into the yeast strains MAY2169 and MAY2371 (Table 1).

To sequence the suppressing alleles of *KAR3*, a *Stu*I-*Pst*I fragment was removed from each suppressing DNA plasmid and inserted into M13 mp19 (YANISCH-PERRON, VIEIRA and MESSING 1985) cut with *Sma*I and *Pst*I. Sequencing of the complete *KAR3* reading frame for each mutant and the wild-type was achieved on one strand in the 3′ to 5′ direction with respect to *KAR3* transcription. The sequence originating at the *Stu*I site was derived using the "universal −40 sequencing primer" and the following primers were used successively to obtain the remainder of the gene:

5′-GCCATACGAGTGTGCTCC-3′
5′-CCAGTTAAATATATGAG-3′
5′-CCTCAAGTTCTTTGATC-3′
5′-GGTGATTTTATTTCTTACC-3′
5′-CTAGTGTTGCCTTTTTCTC-3′

The sequence for the wild-type that we obtained agreed in entirety with that published (MELUH and ROSE 1990). For the seven suppressing alleles, all showed the wild-type sequence at the *kar3-1* position (MELUH and ROSE 1990), demonstrating that the gap-repaired DNA had received information from the genomic locus of the transformed cell. In addition, each suppressing DNA contained a single and different nucleotide change from the wild-type within the region encoding the *KAR3* motor domain.

Deletion alleles of *KAR3*: Plasmid-borne carboxyl-terminal deletion alleles of *KAR3* were generated by subcloning of DNA fragments from *KAR3*. A pMA1235 derivative carrying the *kar3-898* allele was used as the source of the *KAR3* DNA. For *kar3-Δ189*, a *Cla*I-*Bst*BI fragment was inserted into pRS316 cut with *Cla*I generating pMA1278. For *kar3-Δ289*, a *Cla*I-*Hin*dIII fragment was inserted into pRS316 cut with the same enzymes generating pMA1275. Note that *kar3-Δ189* retains the *kar3-898* change, but for *kar3-Δ289* it is removed by the deletion.

Microscopic observation: To determine the distribution of cell morphologies, log phase 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 large-budded if the bud diameter was approximately equal to 2/3 the diameter of the mother cell body. The number of nuclei per cell was determined by observation of chromosomal DNA masses by epifluorescent illumination.

Measurements of karyogamy, meiosis and spindle collapse: Karyogamy proficiency was determined by mixing 2.5×10^7 log-phase cells of each parent and spreading evenly over the surface of a standard 100-mm petri dish containing YPD agar medium. After 5 hr of mating at 26°, the cells were washed from the surface of the agar with water and titered for diploids produced (on SD plus histidine, leucine and uracil) and total cells (on YPD). For some crosses (see Table 4) the titer of cytoductants produced was assayed on rich medium containing glycerol instead of glucose and 5 μg/ml cycloheximide (Sigma). To cut down on contaminating glucose, the yeast extract content was reduced by a factor of two in this medium. Diploid products of these assays were tested for meiosis proficiency by patching cells onto sporulation medium. After 2–3 days, cells were scraped from the agar surface, suspended in water and examined microscopically for the appearance of spores.

The assay for spindle collapse following hydroxyurea synchronization utilized an antibody preparation directed against spindle pole bodies (ROUT and KILMARTIN 1990) and was performed exactly as described previously (SAUNDERS and HOYT 1992).

TABLE 1

Yeast strains

Strain	Genotype
MAY390	*MATα his3-Δ200 leu2-3,112 ura3-52 ade2-101 cyh2 rho°*
MAY399	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY589	*MATa his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY591	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2059	*MATa cin8::LEU2 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2065	*MATα cin8::URA3 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2082	*MATα cin8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2169	*MATa cin8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2174	*MATa cin8::URA3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 cyh2 can1*
MAY2196	*MATa cin8-3 kip1::HIS3 kar3-898 his3-Δ200 leu2-3,112 ura3-52 ade2-101 cyh2*
MAY2199	*MATa cin8-3 kip1::HIS3 kar3-898 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2201	*MATa cin8-3 kip1::HIS3 kar3-899 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2202	*MATα cin8-3 kip1::HIS3 kar3-899 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2219	*MATa cin8::LEU2 kip1::HIS3 kar3-891 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2220	*MATα cin8-3 kip1::HIS3 kar3-891 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2221	*MATa cin8-3 kip1::HIS3 kar3-891 his3-Δ200 leu2-3,112 ura3-52*
MAY2222	*MATa cin8-3 kip1::HIS3 kar3-893 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2226	*MATa cin8-3 kip1::HIS3 kar3-894 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2228	*MATα cin8-3 kip1::HIS3 kar3-897 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2232	*MATa cin8-3 kip1::HIS3 kar3-897 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2233	*MATa cin8-3 kip1::HIS3 kar3-8912 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2237	*MATα cin8-3 kip1::HIS3 kar3-8912 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2238	*MATα cin8-3 kip1::HIS3 kar3-8912 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2268	*MATα kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2314	*MATα cin8-3 kip1::HIS3 kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2324	*MATα cin8-3 kip1::HIS3 kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2325	*MATa cin8-3 kip1::HIS3 kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2331	*MATa cin8-3 kip1::HIS3 kar3-891 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2371	*MATα cin8-3 kip1::HIS3 kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 ade2-101 lys2-801*
MAY2498	*MATα kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2500	*MATα kar3-891 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2505	*MATa kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801 cyh2 rho°*
MAY2506	*MATa kar3-891 his3-Δ200 leu2-3,112 ura3-52 lys2-801 cyh2 rho°*
MAY2606	*MATα kar3-898 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2708	*MATα cin8-3 kip1::HIS3 his3-Δ200 ura3-52 lys2-801 cyh2 rho°*
MAY2709	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 cyh2 rho°*
MAY2710	*MATa kar3-898 CIN8:URA3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 lys2-801 can1 cyh2 rho°*
MAY2746	*MATa cin8::URA3 kip1::HIS3 kar3-102::LEU2 his3-Δ200 leu2-3,112 ura3-52 ade2-101 lys2-801* (pMA1260 = *CIN8* − *LYS2* − CEN)
MAY2821	*MATα cin8-3 kip1::HIS3 kar3-893 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2822	*MATa cin8-3 kip1::HIS3 kar3-893 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2823	*MATa cin8-3 kip1::HIS3 kar3-894 his3-Δ200 leu2-3,112 ura3-52 ade2-101*
MAY2825	*MATα cin8-3 kip1::HIS3 kar3-894 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY2997	*MATa cin8-3 kip1::HIS3 kar3-898 his3-Δ200 leu2-3,112 ura3-52 ade2-101* (pMR798 = *KAR3* − *URA3* − CEN)
MAY3064	*MATα cin8-3 kip1::HIS3 kar3-891 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY3065	*MATα cin8-3 kip1::HIS3 kar3-898 his3-Δ200 leu2-3,112 ura3-52 lys2-801*
MAY3066	*MATα cin8-3 kip1::HIS3 revA2 his3-Δ200 leu2-3,112 ura3-52 lys2-801*

RESULTS

Extragenic suppressors of *cin8 kip1*: The *CIN8* and *KIP1* gene-products apparently overlap in function (HOYT *et al.* 1992; ROOF, MELUH and ROSE 1992). The function of either of these gene-products alone permits normal growth rates (except at the extreme growth temperature of 37° where *cin8* single mutants are inviable). We selected pseudorevertants in a genetic background in which both gene-products were defective. The strain utilized (MAY2082) has the *cin8-3* temperature-sensitive allele and a deletion (*kip1::HIS3*) in which *KIP1* DNA was substituted with *HIS3* DNA (HOYT *et al.* 1992). Strains of this genotype are unable to grow at 33° and higher (Figure 1). Pseudorevertants able to grow at 35° arose at a frequency of approximately one in 2×10^6 individuals. Eight pseudorevertants that arose independently were chosen for further analysis. All grew well at 35° but only one, *revA2*, was able to grow at 37° (Figure 1).

The pseudorevertants were first crossed to a strain

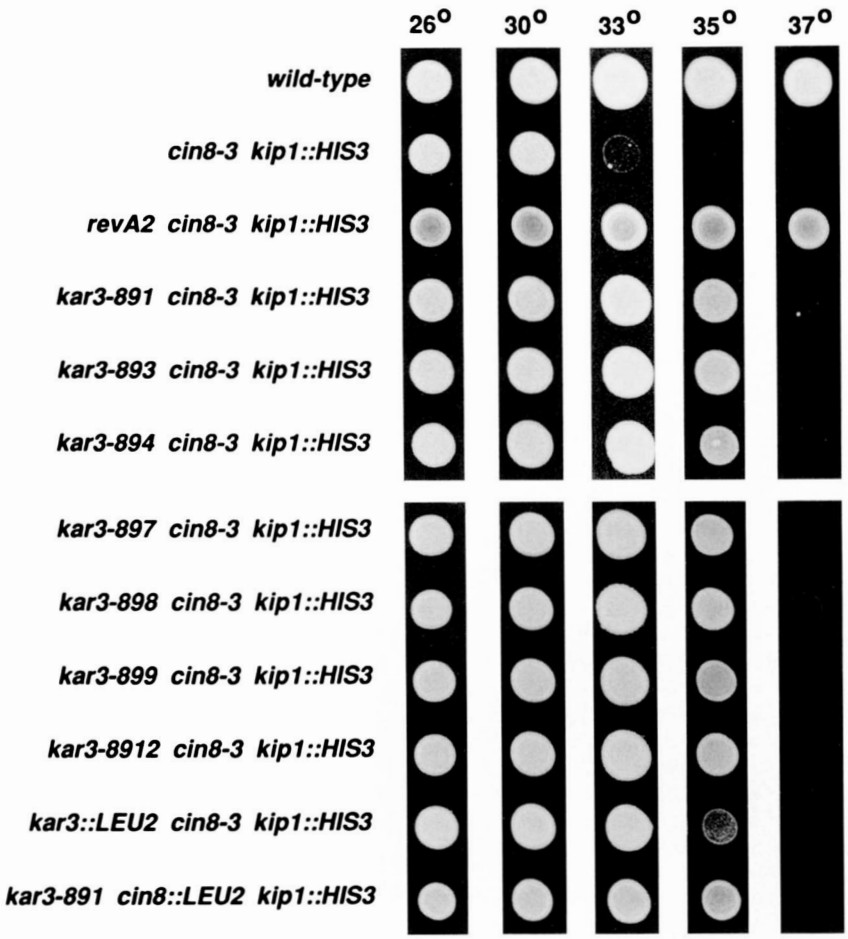

FIGURE 1.—Effect of temperature on growth of strains. Cells of the indicated genotype were resuspended in water, spotted onto rich medium and incubated at the indicated temperature.

(MAY2059) in which *CIN8* DNA had been replaced with *LEU2* DNA (HOYT *et al.* 1992).

Cross 1: *cin8-3 kip1::HIS3 rev* x *cin8::LEU2*,

where *rev* represents the suppressing mutation. Among the haploid progeny of this cross, those with a Leu^- His^+ phenotype possess the same *cin8-3 kip1::HIS3* genotype as the *rev* parent. For the cross using the *revA2* parent, all 13 Leu^- His^+ progeny obtained were able to grow at 35°, indicating that the suppressing mutation is linked to either the parental *CIN8* or *KIP1* loci. It is highly probable that the *revA2* mutation is an intragenic revertant of the *cin8-3* gene since an alteration at the deleted *kip1* locus that suppresses yet retains *HIS3* is unlikely.

For the remaining seven pseudorevertants, the Leu^- His^+ progeny of Cross 1 were distributed evenly between those that could grow at 35° and those that could not. This finding indicated that the suppressing locus was unlinked from either *CIN8* or *KIP1*. In addition, viable Leu^+ His^+ haploid progeny were obtained from all seven of these crosses. These possess a normally inviable genotype in which both *CIN8* and *KIP1* loci are deleted (HOYT *et al.* 1992; ROOF, MELUH and ROSE 1992). Therefore, the suppressing alleles obtained can bypass the requirement for *CIN8* and *KIP1* function. Suppression is not complete, however. The *cin8::LEU2 kip1::HIS3 rev* strains constructed all grew with reduced rates and were temperature-sensitive for viability (see Figure 1 for example).

The suppressing mutations are alleles of *KAR3*: At the time these experiments were initiated, two other kinesin-related genes, *KAR3* and *KIP2*, had been identified in *S. cerevisiae* (MELUH and ROSE 1990; ROOF, MELUH and ROSE 1992). The possibility that the extragenic suppressing mutations were alleles of kinesin-related *KAR3* was tested by the following cross:

Cross 2: *cin8-3 kip1::HIS3 rev* x *CIN8:URA3 kar3::LEU2*,

where *kar3::LEU2* is a deletion of *KAR3* into which *LEU2* DNA has been substituted [*kar3-102::LEU2* from(MELUH and ROSE 1990)] and *CIN8:URA3* is a duplication at the *CIN8* locus marked with *URA3*. Ura^- His^+ haploid progeny from this cross possess the *cin8-3 kip1::HIS3* genotype. Among these, we found that all that were Leu^- possessed the suppressing mutation; they could grow at 35°. This indicated that *LEU2*, which marks the *KAR3* locus, and the suppressing mutations are linked. Two of the extragenic suppressors were tested by Cross 2 and both showed

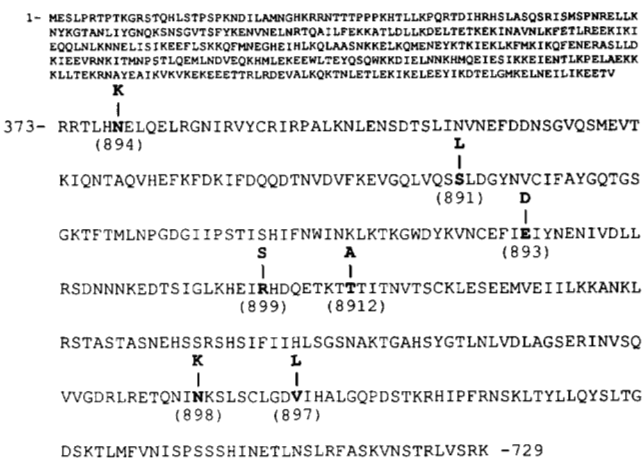

FIGURE 2.—Changes in the Kar3p sequence caused by the suppressing mutations. The complete primary sequence of Kar3p is depicted in single letter code [from MELUH and ROSE (1990)]. Numbers on the side correspond to amino acid positions. The motor domain sequence, identified by its similarity to the corresponding domain from kinesin, is depicted in larger type. Bold type indicates the positions changed in the seven suppressing alleles sequenced. The mutant residue is indicated above the sequence line and the allele number is in parentheses below the line. Codon changes were as follows: *kar3-891*, TCA (ser) to TTA (leu); *kar3-893*, GAG (glu) to GAT (asp); *kar3-894*, AAT (asn) to AAG (lys); *kar3-897*, GTT (val) to CTT (leu); *kar3-898*, AAT (asn) to AAG (lys); *kar3-899*, CGT (arg) to AGT (ser); *kar3-8912*, ACC (thr) to GCC (ala).

linkage to *kar3::LEU2* (all 8 and 13 Ura$^-$ His$^+$ Leu$^-$ progeny tested from rev1a and rev7a crosses, respectively, were suppressed at 35°). The remaining five extragenic suppressors showed 100% linkage to the suppressing locus in rev1a in crosses of the following form:

Cross 3: *cin8-3 kip1::HIS3 revX* x *cin8-3 kip1::HIS3 rev1a*

The observation of genetic linkage between the extragenic suppressors and the *KAR3* locus strongly suggested that suppression might be caused by changes in the *KAR3* gene itself. DNAs containing the mutations for all seven suppressors were obtained by the gap mis-repair method (see MATERIALS AND METHODS) using a *KAR3* plasmid as the recipient. Sequencing of the complete *KAR3* open reading frame revealed only a single, but different, nucleotide change in each of the seven examined (Figure 2). All seven changes were confined to the carboxyl-terminal region of *KAR3* that encodes the domain with strong sequence similarity to the motor domain of the kinesin heavy chain. Since the extragenic (*rev*)suppressors are clearly mutant alleles of *KAR3*, we redesignated them as *kar3-891, -893, -894, -897, -898, -899, -8912.*

In the course of examining the progeny of Cross 2, and a control cross identical except for the absence of the suppressing (*rev*) mutation, we noticed that Ura$^-$ His$^+$ Leu$^+$ progeny (*cin8-3 kip1::HIS3 kar3::LEU2*) were also more temperature-resistant than *cin8-3 kip1::HIS3* strains (Figure 1). Therefore, as reported previously (SAUNDERS and HOYT 1992), a deletion of *KAR3* partially suppresses the temperature sensitivity of the *cin8-3 kip1::HIS3* genotype. The increase in temperature-resistance caused by *kar3::LEU2* is not quite as high as that caused by the seven *kar3* missense alleles (Figure 1, compare 35° spots). In addition, *kar3::LEU2* will not bypass the requirement for either *CIN8* or *KIP1* function for viability. Strains of the genotype *cin8::URA3 kip1::HIS3 kar3::LEU2 lys2* and harboring a plasmid carrying *CIN8* and *LYS2* were constructed (*i.e.*, MAY2746). These strains were unable to segregate cells capable of growth on medium containing α-aminoadipate, a compound that selects against the *LYS2* genotype. This indicated that these strains could not survive following loss of the *CIN8*-containing plasmid. Therefore, the *cin8 kip1 kar3* triple deletion genotype is not viable.

The *kar3* alleles block *cin8/kip1* spindle collapse: When *cin8-3 kip1::HIS3* (*KAR3*) cells are shifted to temperatures nonpermissive for growth, pre-anaphase bipolar mitotic spindles collapse (SAUNDERS and HOYT 1992). Previously separated spindle poles rapidly assume a side-by-side orientation. This collapse is partially inhibited by a deletion of *KAR3*, suggesting that Kar3p contributes to the force that pulls spindle poles toward each other (SAUNDERS and HOYT 1992).

The ability of the suppressing alleles of *KAR3* to block spindle collapse was assessed. Cells were treated at 26° with the DNA synthesis inhibitor hydroxyurea. This caused the cells to arrest their mitotic cycles such that most possessed short spindles (PRINGLE and HARTWELL 1981). The cells were removed to fresh medium minus hydroxyurea and shifted to 33° or 37°. After 10 min, the cells were fixed, treated with an antibody directed against spindle poles (ROUT and KILMARTIN 1990) and observed by immunofluorescence microscopy. Cells with separated spindle poles typically possess two spots of stained material, whereas cells with side-by-side poles possess one (SAUNDERS and HOYT 1992). Following incubation at either of the two elevated temperatures, most spindles in wild-type cells remained bipolar (Table 2). In contrast, after incubation at 37°, less than 10% of the original *cin8-3 kip1::HIS3* bipolar spindles had two separated poles. In agreement with previous experiments (SAUNDERS and HOYT 1992), *kar3::LEU2* partially rescued the spindle collapse phenotype. *kar3-891* also was able to prevent collapse to about the same extent as the deletion allele. *kar3-898* was able to completely block collapse, even at 37°, a temperature that is nonpermissive for growth of this strain (Figure 1). Extended examination of the *kar3-898* cells during incubation at 37° revealed that most were actually able to complete mitosis at this temperature, but arrested in the

TABLE 2

***kar3* alleles block spindle collapse**

Strain	Genotype	Percent cells with separated poles[a] 26°	33°	37°	Percent viable after HU arrest[b]
MAY589	Wild-type	55	55.5	46.5	89
MAY2169	*cin8-3 kip1::HIS3*	51	23	5.5	93
MAY2371	*cin8-3 kip1::HIS3 kar3::LEU2*	59	43.5	33	69
MAY2220	*cin8-3 kip1::HIS3 kar3-891*	63	58	32	90
MAY2199	*cin8-3 kip1::HIS3 kar3-898*	53	49	52	74

[a] Cells of the indicated genotype were synchronized with hydroxyurea at 26° and released at 33° or 37°. Samples taken from before and 10 min after the temperature shift were fixed. These were stained with the antibody directed against the 90 kD SPB component and examined by immunofluorescence microscopy. The percentage of cells with two clearly separated spindle poles was determined. At least 200 cells were counted for each sample.

[b] Cells were synchronized with hydroxyurea at 26° and spread onto solid rich medium minus the inhibitor; 120 cells from each strain were individually micromanipulated to specific positions on the agar surface and the percentage that were able to form colonies at 26° was determined.

next cell cycle during spindle assembly (data not shown).

It has been reported that up to 40% of the cells in a *kar3::LEU2* culture are lethally arrested in mitosis and possess a short spindle (MELUH and ROSE 1990; see below). It was a concern, therefore, that the *kar3* alleles were not directly reducing *cin8 kip1* spindle collapse but that the cells in which collapse did not occur had simply expired at the *kar3* mitotic arrest stage. To distinguish between these possibilities, the abilities of cells of the above genotypes to recover from the hydroxyurea arrest at 26° was determined (Table 2). A reduction in viability relative to *KAR3* was observed for the *kar3::LEU2* and *kar3-898* mutant strains. However, *kar3-891*, which was able to suppress collapse as well as *kar3::LEU2*, did not cause any difference in viability from *KAR3*. This lack of correlation between viability and suppression makes it unlikely that the observed reduction in collapsed spindles is caused by *kar3*-induced mitotic lethality.

Dominance of the suppression phenotype: It is of interest to determine whether the suppressing alleles of *KAR3* represent a loss- or a gain-of-function change with respect to the wild-type (see DISCUSSION). Often, the answer to this problem can be suggested by a dominance test. For a "classical" dominance assessment, diploid strains were constructed that were homozygous for both *cin8-3* and *kip1::HIS3*, but were heterozygous for *KAR3*. All seven *kar3* missense alleles were dominant over *KAR3* for the high temperature suppression phenotype (Table 3) as was the *kar3::LEU2* deletion allele. From the deletion result, we can conclude that in this test a loss of function of *KAR3* is dominant to wild-type. This is not the common expectation from a dominance test where loss of function is typically recessive to the wild-type. In this assay, therefore, part or all of the dominance for suppression could reflect lowered gene-product activity.

TABLE 3

Dominance of the suppression phenotype

kar3 allele	Diploid test[a]	Haploid test[b]
-Δ	Y	N
-891	Y	N
-893	Y	N
-894	Y	Slight
-897	Y	N
-898	Y	Y
-899	Y	Slight
-8912	Y	N

[a] Diploid strains were constructed that were homozygous for *cin8-3* and *kip1::HIS3* and were heterozygous for the indicated *kar3* allele over *KAR3*. The Y indicates that all grew approximately as well at 33° and 35° as a strain homozygous for *cin8-3*, *kip1::HIS3* and the indicated *kar3* allele.

[b] MAY2169, a haploid *cin8-3 kip1::HIS3* (*KAR3*) strain, was transformed with a plasmid carrying the indicated *kar3* allele (or vector only for -Δ). Y indicates the strain grew as well at 33° and 35° as a *cin8-3 kip1::HIS3* haploid with the indicated allele at the *KAR3* chromosomal locus. At these temperatures, the untransformed parent strain did not grow. N indicates that there was no observed difference in temperature-resistance between the transformed haploid and the untransformed parent strain and "slight" indicates a very slight increase in temperature-resistance after transformation.

We performed a second dominance test that differed from the above diploid test in the relative gene dosage for *KAR3*. In this test a *kar3* null allele could not score as dominant for suppression. A *cin8-3 kip1::HIS3* (*KAR3*) haploid strain was transformed with low copy (centromere) plasmids containing the various suppressing alleles. A vector-only transformant served as the *kar3* null control and, as expected, displayed no increase in temperature-resistance. In this haploid cell assay, four of the seven missense alleles that were dominant in the diploid assay now showed no effect on temperature-resistance (Table 3). Two alleles, *kar3-894* and *-899*, showed a very slight dominance and only one, *kar3-898*, showed clear dominance for temperature-resistance.

Suppression requires the motor domain of Kar3p:

It is possible that suppression by the mutant forms of Kar3p is caused by simple inactivation of the motor domain. The amino-terminal "tail" of the protein acting alone might suppress. To test this possibility, we constructed two deletions that removed various amounts of the motor domain and tested these for suppressing activity. The deletions were made from centromere plasmid DNA containing the dominant suppressor *kar3-898*. *kar3-898* differs from *KAR3* at the codon encoding amino acid 650 (Figure 2). *kar3-Δ189* removes all amino acids following position 693 and therefore retains the *kar3-898* change. *kar3-Δ289* removes all amino acids following position 568 and therefore removes the *kar3-898* change but still retains the whole amino-terminal tail domain [the motor begins at about position 373 (MELUH and ROSE 1990)].

The introduction of plasmids carrying the two *KAR3* deletions into MAY2169, a *cin8-3 kip1::HIS3* strain, did not cause any increase (or decrease) in temperature-resistance. Therefore, both of these deletions destroyed the dominant suppressing activity observed for *kar3-898*. When transformed into MAY2371, a *cin8-3 kip1::HIS3 kar3::LEU2* strain, again no change in temperature-resistance was observed. This indicated that these deleted forms of *kar3-898* could suppress no better than a deletion of the complete *KAR3* reading frame.

Effects of new *kar3* alleles on mitosis, meiosis and karyogamy: *KAR3* deletion mutations cause a partial mitotic defect; asynchronous mutant cultures accumulate abnormally high numbers of large-budded mononucleate cells, a morphology characteristic of mitosis delay or arrest (MELUH and ROSE 1990). Two of the new *KAR3* alleles were crossed into *CIN8 KIP1* backgrounds. These strains, as well as a *KAR3* strain and a deletion strain were grown to log phase, fixed, stained for DNA and observed microscopically. Cultures of the *KAR3* and *kar3-891* strains (MAY591 and MAY2500, respectively) both yielded 2.5% cells with a large-budded, mononucleate morphology. The *kar3::LEU2* strain (MAY2268) yielded 34% and *kar3-898* (MAY2606) yielded 18.3%. Therefore, the *kar3-898* allele causes a partial mitotic defect that is less severe than that caused by *kar3::LEU2*.

KAR3 function is essential for the processes of karyogamy and meiosis (POLAINA and CONDE 1982; MELUH and ROSE 1990; P. MELUH and M. ROSE, unpublished data). The *KAR3* alleles that suppress *cin8/kip1* defects were tested for their ability to support these two processes (Table 4). To assay karyogamy, the efficiency of diploid production was measured in matings between parents with different *KAR3* alleles. For some matings, we also assayed the production of cytoductants. Cytoductants are haploid cells that result from karyogamy failure, with cytoplasm contributed by both parents but the nucleus of only a single parent. To assay meiosis, diploid cells of various *KAR3* genotypes were patched onto sporulation medium and observed microscopically after 3 days for the appearance of spores. All *KAR3* alleles were assayed in the *cin8-3 kip1::HIS3* background. *kar3-891* and *kar3-898* were also assayed in the *CIN8 KIP1* background. The presence of the *cin8-3*, *cin8::URA3* and *kip1::HIS3* mutations were found to have no effect on karyogamy and meiosis efficiencies under the conditions tested (Table 4).

With the exception of *kar3-898*, none of the suppressor alleles of *KAR3* showed any effect on either karyogamy or meiosis (Table 4). In contrast, *kar3-898* homozygous diploids produced no visible spores on sporulation medium. Mating two *kar3-898* parents resulted in a large decrease in karyogamy efficiency, although not as large as mating two *kar3::LEU2* parents. As has been reported for *kar3::LEU2* (MELUH and ROSE 1990), the karyogamy defect caused by *kar3-898* is bilateral; only when both parents were mutant was an effect on karyogamy observed (Table 4). In addition, *kar3-898* is recessive for both the karyogamy and meiosis defects. The introduction of a centromere plasmid carrying wild-type *KAR3* to one parent restored karyogamy and meiosis to normal levels.

DISCUSSION

Kinesin-related Cin8p and Kip1p are required for an outwardly directed force that acts upon the spindle poles (HOYT *et al.* 1992; ROOF, MELUH and ROSE 1992). In their absence, separated spindle poles are rapidly drawn back together (SAUNDERS and HOYT 1992). The observation that a deletion of kinesin-related *KAR3* partially suppressed this defect led to the conclusion that Kar3p function antagonizes Cin8p/Kip1p function, perhaps by contributing to the inwardly directed force (SAUNDERS and HOYT 1992). Kar3p is essential for the processes of karyogamy and meiosis (POLAINA and CONDE 1982; MELUH and ROSE 1990; P. MELUH and M. ROSE, unpublished data). It also performs an important, but nonessential role in mitosis. *kar3* deletion mutant cultures are characterized by an abnormally high number of cells in the mitotic phase and many inviable cells that appear to have expired during mitosis (MELUH and ROSE 1990). Note that although all three proteins possess kinesin motor domain-like regions, Kar3p and Cin8p/Kip1p have strikingly different structures. Kar3p, like the more closely related klpA and ncd proteins of *Aspergillus* and *Drosophila* respectively, has its motor domain at the carboxyl terminus of the polypeptide (MELUH and ROSE 1990; O'CONNELL *et al.* 1993; ENDOW, HENIKOFF and SOLER-NIEDZIELA 1990; MCDONALD and GOLDSTEIN 1990). Cin8p and Kip1p,

TABLE 4

Karyogamy and meiosis proficiency of the suppressing alleles

Mated strains[a]	Genotype[b]	% Diploids[c]	C/D[d]	Meiosis[e]
390 × 399	+	68	1.9×10^{-3}	+
2498 × 2505	*kar3::LEU2*	0.036	6.7	−
2500 × 2506	*kar3-891*	65	1.6×10^{-3}	+
2606 × 2710	*kar3-898*	1.4	7.9	−
2606 × 2709	*kar3-898* × +	65	4.9×10^{-3}	+
2169 × 2708	*cin8-3 kip1::HIS3*	75	3×10^{-3}	+
589 × 591	+	70		+
2065 × 2174	*cin8::URA3*	71		+
2314 × 2325	*cin8-3 kip1::HIS3 kar3::LEU2*	0.011		−
2331 × 3064	*cin8-3 kip1::HIS3 kar3-891*	80		+
2821 × 2822	*cin8-3 kip1::HIS3 kar3-893*	76		+
2823 × 2825	*cin8-3 kip1::HIS3 kar3-894*	64		+
2228 × 2232	*cin8-3 kip1::HIS3 kar3-897*	74		+
2201 × 2202	*cin8-3 kip1::HIS3 kar3-899*	54		+
2233 × 2237	*cin8-3 kip1::HIS3 kar3-8912*	64		+
2196 × 3065	*cin8-3 kip1::HIS3 kar3-898*	1.6		−
3065 × 2997	*cin8-3 kip1::HIS3 kar3-898* × *cin8-3 kip1::HIS3 kar3-898* (pKAR3)[f]	60		+

[a] MAY strain numbers for the two mated strains. See Table 1 for full genotypes.

[b] Relevant genotype for mated strains. When only one genotype is indicated, both parents were the same. When the parents differed, both genotypes are indicated.

[c] The percentage of total cells (colony forming units on rich medium) that can form colonies on minimal medium selective for diploids.

[d] Cytoductant titer divided by the diploid titer. Cytoductants determined as titer of cells that could form colonies on glycerol-cycloheximide plates (see MATERIALS AND METHODS).

[e] + indicates that after incubation on sporulation medium, at least 15% of the cells observed microscopically contained three or four clear spore bodies. − indicates that no clear spores could be found in at least 1,000 cells observed.

[f] Indicates the presence of pMR798, a *KAR3*-containing plasmid.

like kinesin and most other characterized relatives, have their motor domains at the amino termini (HOYT *et al.* 1992; ROOF, MELUH and ROSE 1992).

Further support for a mitotic role for Kar3p is provided by the results reported herein. Missense mutant alleles of *KAR3* were identified that could suppress the mitotic inviability of *cin8 kip1* mutants. All seven missense alleles analyzed possessed properties that were different from the *kar3* deletion. Each suppressed the temperature-sensitivity of the *cin8-3 kip1::HIS3* genotype better than the *kar3* deletion and in addition could bypass the requirement for *CIN8 KIP1*. Six of the seven were also unaffected for their karyogamy and meiosis properties and the seventh was dominant for its suppressing activity. These differences demonstrate the expression of a functional gene-product by these mutant *kar3* genes.

The changes in *KAR3* that result in suppression were all found to have occurred in the motor domain-encoding region. It is possible that suppression was achieved by complete inactivation of motor domain function. However, constructed deletions of motor domain-encoding sequences did not result in suppression. Assuming that the truncated forms of Kar3p were not significantly altered in expression levels or stability, we conclude that complete elimination of motor domain function cannot suppress.

It is important to consider whether the *KAR3* missense alleles suppress because they cause a Kar3p gain of function that allows them to substitute for Cin8p/Kip1p, or they cause a loss of function that eliminates an activity antagonistic to Cin8p/Kip1p. The suppression by the *kar3* deletion clearly indicates that loss of function can contribute to suppression. However, properties of the missense alleles suggested that they may represent a gain of function; some were dominant for suppression and all could bypass the requirement for Cin8p/Kip1p function. Below we consider the possibility that the suppressing alleles cause a partial loss of Kar3p activity that may in effect confer a new functional property on Kar3p.

When assayed in diploid cells, the *kar3* deletion and missense alleles were dominant for the suppression phenotype. The dominance exhibited here is most simply interpreted as caused by lowered Kar3p activity; diploid cells must require two functional copies of *KAR3* for a normal level of activity. In haploid cells, the deletion, as well as four of the missense alleles, no longer displayed any dominance. For these *kar3* alleles, this finding is consistent with a reduction in Kar3p mitotic activity. The one *kar3* allele that was clearly dominant for suppression, *kar3-898*, was in addition the only allele isolated that was defective for karyogamy and meiosis. *kar3-898* was recessive for these defects. In addition, *kar3-898* caused a partial mitotic defect. The *kar3-898* protein probably has not

gained an activity not intrinsic to the wild-type. Most likely, the altered protein has lost activity and in addition can interfere with the mitotic function of the wild-type protein.

Perhaps the most significant property of the *kar3* missense alleles is their ability to suppress the *cin8 kip1* double deletion. We suggest two possible explanations for this effect. First, the altered Kar3ps may not only be deficient for activity, but also may act to interfere with another activity that like Kar3p, antagonizes Cin8p/Kip1p function. For the second and more likely possibility, Kar3p and Cin8p/Kip1p may function in a similar manner, despite their apparent ability to act antagonistically. A shared property may be the ability to crosslink spindle midzone microtubules, as has been proposed for Cin8p/Kip1p (SAUNDERS and HOYT 1992). The identification of a distinct microtubule-binding domain outside of the motor domain suggests that Kar3p may be able to crosslink microtubules (MELUH and ROSE 1990). In this formulation, Kar3p antagonism with Cin8p/Kip1p could be achieved if these motors generated a sliding force between cross-linked microtubules, but in opposing directions. The suppressing missense alleles of *KAR3* may act by inactivating the antagonistic motor function, but retaining the microtubule-crosslinking property. Consistent with this view, a deletion of *KAR3*, which would remove both the antagonistic and the crosslinking functions, would not be able to replace *CIN8* and *KIP1*. In considering either of these possibilities, one is led to the conclusion that an unidentified Cin8p/Kip1p-like activity must be operating. Although it is possible that the missense alleles of *KAR3* convert the product into a Cin8p/Kip1p-like motor that can substitute in function, the extreme differences in the primary structures of these proteins make this seem unlikely.

Implications for activities of kinesin-related motors: Members of the kinesin-related superfamily all possess a conserved 340 amino acid motor domain (between 29–65% identity). In kinesin, this domain is capable of generating force along microtubules at the expense of ATP (YANG *et al.* 1990; GOLDSTEIN 1991). The seven alleles of *KAR3* described herein all alter this motor domain and therefore identify residues that may be important for its function. Notably, the seven resulting changes do not cluster in one particular region but are distributed throughout the motor domain (Figure 2). Changes in a protein that generally reduce activity may be expected to be less prone to cluster relative to changes that confer a new activity on the protein. Therefore, this finding is consistent with the above hypothesis that the *kar3* alleles cause a partial loss of function.

Three of the *kar3* alleles change amino acid residues that are highly conserved within the kinesin superfamily. Both *kar3-893* and *kar3-898* change residues that are invariant among currently identified superfamily members. The *kar3-898* change (asparagine to lysine) caused a dramatic reduction in activity for the two processes for which Kar3p activity is essential, karyogamy and meiosis. However, the *kar3-893* change (glutamate to aspartate) had no apparent effect on these processes. *kar3-897* changes a valine (to a leucine) that is conserved in 21 of 25 kinesins and related proteins found in current databases. This change also had no effect on karyogamy and meiosis. The conservative *kar3-893* and *kar3-897* changes may simply reduce motor activity enough to suppress *cin8 kip1*, but not enough to affect karyogamy or meiosis. The *kar3-894* mutation changes an asparagine (to lysine) located at the amino terminus of the motor domain. This residue is part of a motif that is highly conserved, but only between Kar3p and its two close relatives, the klpA and ncd proteins (O'CONNELL *et al.* 1993). This motif may be required for a motor activity that is specific to this subgroup of kinesin relatives.

Combining our findings with those of others reveals a variety of phenotypes possible for different *KAR3* alleles (POLAINA and CONDE 1982; MELUH and ROSE 1990; ROOF, MELUH and ROSE 1991). The currently identified alleles are differentially affected for karyogamy (also, bilateral *vs.* unilateral), meiosis and *cin8 kip1* suppression and also differ with respect to their dominance for the defect caused. Perhaps such phenotypic variety can be expected for a protein that apparently has distinct functional domains and that participates in three distinct cellular processes.

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