Localization of the Kar3 Kinesin Heavy Chain-related Protein Requires the Cik1 Interacting Protein

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Abstract. The Kar3 protein (Kar3p), a protein related to kinesin heavy chain, and the Cikl protein (Ciklp) appear to participate in the same cellular processes in S. cerevisiae. Phenotypic analysis of mutants indicates that both CIK1 and KAR3 participate in spindle formation and karyogamy. In addition, the expression of both genes is induced by pheromone treatment. In vegetatively growing cells, both Ciklp::β-gal and Kar3p::β-gal fusions localize to the spindle pole body (SPB), and after pheromone treatment both fusion proteins localize to the spindle pole body and cytoplasmic microtubules. The dependence of Ciklp and Kar3p localization upon one another was investigated by indirect immunofluorescence of fusion proteins in pheromone-treated cells. The Ciklp::β-gal fusion does not localize to the SPB or microtubules in a kar3Δ strain, and the Kar3p::β-gal fusion protein does not localize to microtubule-associated structures in a cik1Δ strain. Thus, these proteins appear to be interdependent for localization to the SPB and microtubules. Analysis by both the two-hybrid system and co-immunoprecipitation experiments indicates that Ciklp and Kar3p interact, suggesting that they are part of the same protein complex. These data indicate that interaction between a putative kinesin heavy chain-related protein and another protein can determine the localization of motor activity and thereby affect the functional specificity of the motor complex.

Microtubules participate in a wide variety of cellular processes, including mitosis, organelle transport, and cell motility. These processes are mediated by microtubule motors, often kinesin-related proteins (Vale et al., 1985a; Zhang et al., 1990; Enos and Morris, 1990; Meluh and Rose, 1990; McDonald et al., 1990; Walker et al., 1990; Roof et al., 1992a; Hoyt et al., 1992; Hagan and Yanagida, 1990; Hagan and Yanagida, 1992). Kinesin was originally identified as a tetrameric complex comprised of two heavy chain subunits and two light chains (Vale et al., 1985a). The heavy chain contains a motor domain that mediates translocation of the motor along microtubules (Yang et al., 1989); the function of the light chain is unknown.

Recently, a large number of kinesin heavy chain-related molecules have been found and characterized (Vale et al., 1985a; Zhang et al., 1990; Enos and Morris, 1990; Meluh and Rose, 1990; McDonald et al., 1990; Walker et al., 1990; Roof et al., 1992a; Hoyt et al., 1992; Hagan and Yanagida, 1990; Hagan and Yanagida, 1992). These proteins often contain a central coiled-coil domain flanked by a common motor domain and a nonconserved globular domain. Despite the similarities among the different members of the kinesin family, the various motor proteins participate in many different functions (Vale et al., 1985b; McDonald et al., 1990; Zhang et al., 1990; Saxton et al., 1991; Saunders and Hoyt, 1992; O'Connell et al., 1993; Goldstein, 1990). The specificity of function has been speculated to be conferred in part by the nonhomologous region of these proteins (Yang et al., 1989; Meluh and Rose, 1990; Enos and Morris, 1990; Vale and Goldstein, 1990). Through its interaction with light chains or other proteins, this region may determine the localization of the microtubule motor, affect its activity, and/or specify the type of cargo carried (Vale et al., 1985a; Cyr et al., 1991; Wedaman et al., 1993). So far no direct evidence supports this hypothesis.

The yeast Saccharomyces cerevisiae is an excellent system for the genetic analysis of microtubule-dependent processes such as chromosome segregation during mitosis and nuclear fusion (karyogamy) during conjugation. Molecular characterization of several mutants defective in these processes has uncovered a family of genes encoding kinesin-related proteins. These genes include KIP1 and CIN8 which participate in the establishment and maintenance of the mitotic apparatus and KAR3 which also functions in chromosome segregation and is required for karyogamy (Hoyt et al., 1992; Roof et al., 1992b; Meluh and Rose, 1990). Genetic screens that enrich for mutants defective in microtubule processes have identified other genes whose products interact with microtubules but share no or little sequence similarity to previously
characterized proteins (Berlin et al., 1990; Page and Snyder, 1992). Such genes could encode proteins that function via cooperation with kinesin-like motors. Mutations in such a gene would be expected to result in similar phenotypes to those exhibited in cells mutant for the corresponding kinesin-related gene. Given this assumption, the Cikl gene product is a candidate for interacting with the Kar3 kinesin-related protein.

Cells deleted for either CIKI or KAR3 exhibit similar phenotypes (Meluh and Rose, 1990; Page and Snyder, 1992). Neither gene is essential for vegetative growth, and mutations in either cause defects in chromosome stability and establishment/maintenance of the mitotic spindle apparatus. ciklΔ and kar3Δ strains have more prominent microtubule arrays than wild-type cells and both exhibit severe defects in karyogamy. In addition, the expression of both CIKI and KAR3 is induced by treatment with mating pheromone. Kar3p is a kinesin-related protein; its sequence predicts a central coiled-coil domain with a putative microtubule motor domain at the carboxy terminus. Ciklp is not homologous to microtubule motor proteins; however, it does have a putative central coiled-coil domain. Ciklp:β-galactosidase (β-gal) fusion proteins have been localized to the spindle pole body (SPB) and along microtubules in vegetative cells; a Kar3p:β-gal fusion lacking the motor domain exhibits a similar localization pattern in pheromone-treated cells. In addition to demonstrating the similar localization patterns of these two proteins, this latter observation indicates that the nonmotor domain of Kar3p directly or indirectly associates with microtubules.

In this article, the phenotypic similarities between ciklΔ and kar3Δ cells are examined in greater detail, and the results indicate that the corresponding proteins appear to function in the same pathway. Ciklp and Kar3p are shown to interact by the two hybrid system (Fields and Song, 1989) and by co-immunoprecipitation experiments, suggesting that they are part of the same protein complex. Furthermore, Ciklp and Kar3p are interdependent for localization to the SPB and microtubules in pheromone-treated cells. We speculate that Ciklp helps mediate the specialized function of the Kar3p motor complex by interacting with the nonmotor domain and controlling its localization within the cell.

Materials and Methods

Strains, Media, and Microbiological Techniques

Yeast strains are listed in Table I. General genetic manipulations and growth media were as described (Sherman et al., 1986). Yeast transformations were performed by the lithium acetate procedure (Ito et al., 1983). β-gal assays were performed on yeast using a protocol described for E. coli (Sambrook et al., 1989). General molecular cloning techniques were as described (Maniatis et al., 1982).

Plasmid Constructs

The cikl-6::lacZ LEU2 URA3 CEN plasmid (pB20) was described in Page and Snyder (1992). The kar3-3::lacZ fusion was constructed using a KAR3 URA3 CEN plasmid. This plasmid was digested at its single MluI site within the KAR3 coding region, the ends were filled with the large fragment of DNA polymerase and dNTPs. A 3-kb BamHI fragment containing the lacZ gene was isolated from the mini-lacZ::LEU2 transposon (Siefer et al., 1986); it ends were filled in and the resulting fragment inserted into the KAR3 vector that had been treated with calf alkaline phosphatase. The XbaI fragment lacks the 5' sequences necessary for transcription and translation in both E. coli and S. cerevisiae. In the proper orientation, this construct would result in an in-frame fusion between KAR3 and lacZ. Orientation was determined by restriction mapping and fusion in the proper reading frame was confirmed by β-galactosidase assays in yeast. This construct contains exactly the same amount of KAR3 upstream of lacZ as the kAR3::lacZ fusion designed by Meluh and Rose (1990) and appears to function identically.

To construct the lexA::cikl fusions, the lexA vector, pSH2-1 (Fields and Song, 1989; Golemis and Brent, 1992), was digested at EcoRI; this site was filled in and treated with phosphatase. To make the fusion plasmid, p1027, was provided by Dr. R. Brent (Brent and Ptashne, 1985). The KAR3 deletion was generated through two cloning steps. First, the 3.5-kb Xhol–PstI filled-in CIK1 fragment or the 0.56-kb Xhol–HpaI filled-in CIK1 fragment was inserted into the modified lexA EcoRI site to generate p46 or p47, respectively. Orientation was determined by restriction mapping, and the proper reading frame was confirmed by DNA sequencing. The construction of the GALA::kar3 fusion required an additional step. The 1.5-kb XhoI–PstI filled-in CIK1 fragment or the 0.56-kb XhoI–HpaI filled-in CIK1 fragment was inserted into the modified lexA EcoRI site to generate p46 or p47, respectively. Orientation was determined by restriction mapping and fusion in the proper reading frame was confirmed by DNA sequencing. The construction of the GALA::kar3 fusion plasmid, pI027, was provided by Dr. R. Brent and Ptashne, 1985.

Disruption of KAR3

The kar3-3::lacZ plasmid (pB20) was digested with BglII and the vector DNA was purified from the 1.5-kb BglII fragment of the KAR3 coding region. The BglII sites of the vector were filled in and treated with phosphatase. The 1.3-kb XhoI–PstI filled-in fragment was inserted into the lexA::cikl plasmid (p46 or p47) at the XhoI site to generate p48. The expression of the fusion was confirmed by β-galactosidase assays and fusion in the proper reading frame was confirmed by DNA sequencing.
phatase. A 1.1-kb HindIII fragment containing URA3 (derived from YEp24) was added into and inserted into the modified BigII site of the vector, thereby replacing most of the KAR3 coding sequence. A construct in which URA3 transcription is oriented opposite to that of KAR3 was digested with Sall and BamHI and used to replace the KAR3 allele.

**Strain Constructions**

**MA1 kar3Δ and MA1 kar3Δ cikiΔ** strains were constructed by transforming the kar3Δ4::URA3 allele into diploid strains Y1131 (a CIKI/CIKI strain) and Y1119 (a cikiΔ3::LEU2/CIKI strain). These kar3Δ/ kar3Δ diploids were sporulated and subjected to tetrad analysis. For both strains, a typical tetrad generated two large colonies and two smaller colonies. The URA3 marker always cosegregated with the small colony phenotype, indicating that kar3Δ affects cellular growth (for each strain, eight tetrads were evaluated).

The cikiΔ kiplA cin3-3 strain was derived through a series of steps. A kiplΔ kiplA cin3-3/CIN8 diploid was generated by mating Y815 and Y817 (these strains were kindly provided by A. Hoyt laboratory, Johns Hopkins University, Baltimore, MD) and visually selecting zygotes. Diploids were confirmed as such by verifying that single colony isolates were nonmaters. The diploid strains were transformed with the cikiΔ3::LEU2 allele (this deletion allele is described in Page and Snyder, 1992). The transformed strain (Y1139) was sporulated and subjected to tetrad analysis. Eight tetrads were evaluated; within these eight tetrads, six cikiΔ kiplA cin3-3 strains were recovered.

**Immunofluorescence Microscopy**

Indirect immunofluorescence of yeast cells was performed as described (Adams and Pringle, 1984). Strains with plasmids were grown under selective conditions to 2.5 × 10^7 cells/ml, pelleted by centrifugation, resuspended in YPD, and grown for 3 h before treating with pheromone or harvesting. For most experiments, samples were stained with rat anti-tubulin monoclonal antibody YOLI/34 (Kilmartin et al., 1982; obtained from Seralab) and rabbit anti-β-galactosidase antibody (Cappel Laboratories, Malvern, PA) that had been preadsorbed with fixed and permeabilized yeast cells before use to remove nonspecific antibodies. For detection of anti-tubulin antibodies and anti-β-gal antibodies, FITC-conjugated goat anti-rabbit antibodies (Cappel Laboratories) and Texas red-conjugated goat antirabbit antibodies (Amersham Corp., Arlington Heights, IL) were used, respectively. For the experiment shown in Fig. 9, a mouse anti-β-gal monoclonal antibody was used (Promega, Madison, WI). Affinity-purified anti-Ciklp antibodies were prepared as described previously (Page and Snyder, 1992). To increase the immunofluorescent signal for the anti-Ciklp antibodies, the biotin-streptavidin amplification system was used (Page and Snyder, 1992). The fluorescent DNA-specific dye, Hoechst 33258, was used to visualize yeast nuclei and mitochondria.

To ensure that staining of fusion and nonfusion proteins was specific, we performed several types of control experiments which were similar to those described previously (Page and Snyder, 1992). Double immunofluorescence experiments were performed (a) on strains without Kar3p:β-gal or Ciklp:β-gal fusions or (b) without the β-gal primary antibody. In each case no Texas red staining was evident. Thus, the signals are specific for the fusion proteins and are not due to cross-reactive secondary antibodies.

**α-Factor Arrest**

**MA1** cells grown to a mid-log phase in YPD were arrested by adding α-factor (Sigma Chemical Co., St. Louis, MO) to a final concentration of 4 μg/ml. An hour later the same amount of α-factor was added again. After an additional 50 min the culture was examined to evaluate the response. If 80% or more of the cells had formed shmooes, the cells were harvested for immunofluorescence.

**Immunoprecipitations**

A wild-type strain (MS10) and a kar3Δ strain (MS524) were grown to an approximate OD600 of 0.4. Cultures were divided and incubated in the presence or absence of 1 μg/ml α-factor (final concentration; Bachem, Torrance, CA) for 2 h at 37°C. Formation of mating projections was verified by light microscopy. Cells in a 10-ml aliquot of each culture were collected by centrifugation, and resuspended in 300 ml ice-cold modified RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, containing 1 mM Na3P04, 0.2 mM Na β-glycerophosphate, pH 7.0, 0.01 mM Mn3VO4, 5 mM NaF, 1 mM PMSE, and 10 μg/ml each chymostatin, leupeptin, aprotonin, and pepstatin). Cells were lysed with chilled glass beads (0.75 mmol) and the supernatant removed. The beads were washed with 200 ml RIPA buffer, and the wash and lysate combined.

To immunoprecipitate Kar3p, ~400 μl lysate was brought to 1.0 ml with RIPA buffer, 6 μl rabbit polyclonal antiserum to a TrpE fusion of the COOH-terminal domain of Kar3p was added, and the mixture incubated for 90 min. A 1:1 slurry of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and PBS (50 μl) was added and incubated for an additional 45 min. Protein A-Sepharose–antibody complexes were collected at 12,800 g for 1 min, washed twice with 1.0 ml RIPA buffer and once with 1.0 ml 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, that contained the phosphatase and protease inhibitors in modified RIPA (above). The final pellet was resuspended in 50 μl 4X gel sample buffer, boiled for 4 min, and analyzed on a 10% SDS-polyacrylamide gel. For immunoblotting analysis, proteins were transferred to ProBlott membrane (Applied Biosystems, Inc., Foster City, CA). Membranes were blocked in buffer A (150 mM NaCl, 100 mM Tris-HCl, pH 7.4) containing 5% dry milk overnight at 25°C.

To detect protein, membranes were incubated with either affinity-purified anti-Ciklp antibodies (Page and Snyder, 1992) or an IgG fraction of polyclonal antiserum to the Kar3p COOH-terminal domain, washed in buffer A, and incubated with a donkey anti-rabbit IgG-HRP conjugate (Amersham Corp.). Blots were developed using the enhanced chemiluminescence detection kit according to manufacturer's instructions (Amersham Corp.).

**Results**

**The Karyogamy Phenotype of cikiΔ Resembles that of kar3Δ**

The expression of both CIKI and KAR3 is dramatically induced in response to the mating pheromone α-factor, and cikiΔ and kar3Δ mutants have several defects in common. Through additional characterization of cikiΔ mutants and Ciklp, a number of other CIKI-KAR3 similarities emerged. cikiΔ and kar3Δ mutants both exhibit a severe bilateral mating defect (i.e., very few diploids are formed in matings between two mutant cells) (Meluh and Rose, 1990; Page and Snyder, 1992). During mating of two wild-type cells, the nucleus of each partner migrates proximal to the site of cellular fusion; the two SPBs are oriented toward this fusion site and face one another (Byers and Goetsch, 1975; Meluh and Rose, 1990; Berlin et al., 1990; Cross et al., 1988; Rose, 1991). After cell wall breakdown and cytoplasmic fusion, a microtubule bridge forms between the two SPBs and the nuclei move toward each other and fuse (Fig. 1). Previous studies demonstrated that cikiΔ cells failed to fuse their nuclei during conjugation (Page and Snyder, 1992). To examine this nuclear fusion defect more closely, conjugating cikiΔ cells were stained with anti-tubulin antibodies. As shown in Fig. 1, the SPBs of the two respective nuclei orient toward each other, yet the bridge of microtubules between the SPBs does not form (Fig. 1). Instead, the cytoplasmic microtubules of each SPB appear distinct and unconnected with those of the other (200 cells examined). This phenotype is similar to that described for kar3Δ cells (Meluh and Rose, 1990).

In Pheromone-treated Cells, Ciklp::β-gal Localizes Along Cytoplasmic Microtubules

In yeast cells treated with mating pheromone, a Kar3p:β-gal fusion localizes at the SPB and along microtubules (Meluh and Rose, 1990). We therefore analyzed the localization of a Ciklp:β-gal fusion in cells incubated under similar conditions. In vegetative cells, Ciklp:β-gal fusions are detected at the SPB; weak staining is apparent along nuclear and cytoplasmic microtubules (Page and Snyder, 1992). Previously
reported experiments indicated that $C I K I$ expression is induced 20-fold in cells treated with mating pheromone (Page and Snyder, 1992). Thus, we expected that the Ciklp::β-gal localization pattern might be more striking in pheromone-treated cells.

$M A T a$ cells containing a cikl::lacZ fusion were treated with α-factor, which causes cells to arrest in G1 and induces specific conjugation functions (Rose, 1991). The cikl::lacZ fusion in this strain encodes the amino terminal 80% of the protein (amino acid residues 1–489) and includes the putative coiled-coil domain. The pheromone-treated cells were then subjected to immunofluorescence with anti-β-gal antibodies. As shown in Fig. 2, SPB/microtubule staining is readily apparent in greater than 90% of the treated cells ($n > 1,000$ cells). The Ciklp::β-gal fusion localizes along cytoplasmic microtubules, with more pronounced staining often detected at the SPB and at the end of a microtubule bundle distal to the SPB. This staining pattern is much more intense and slightly different from that of Ciklp::β-gal in vegetative cells (see below, Fig. 7) and from that of the authentic Ciklp localization detected in pheromone-treated cells (see below, Fig. 9A). Nevertheless, the cikl::β-gal localization pattern under pheromone-treatment indicates that Ciklp associates with microtubules (see Discussion). In addition, this Ciklp::β-gal localization pattern is very similar to that reported for a Kar3p::β-gal fusion under identical conditions (Meluh and Rose, 1990).

After α-factor treatment a Kar3p::β-gal fusion not only localizes to cytoplasmic microtubules, but it also affects their assembly/disassembly dynamics (Meluh and Rose, 1990). In cells expressing a Kar3p::β-gal fusion, microtubules appear to be more stable as determined by their increased resistance to depolymerization by nocodazole. To evaluate whether the Ciklp::β-gal fusion also possesses this microtubule stabilizing phenotype, $M A T a$ cells containing this fusion were arrested with α-factor and then treated with 15 µg/ml nocodazole (Meluh and Rose, 1990). In control experiments, either cells lacking the Ciklp::β-gal fusion were treated as described above, or cells containing the fusion were subjected to α-factor and nocodazole treatment simultaneously. In Ciklp::β-gal fusion strains that are treated first with pheromone and then nocodazole, a long cytoplasmic microtubule bundle is detected and Ciklp::β-gal is localized along this bundle (Fig. 3). In contrast, in each of the controls, no long microtubules are observed, and the only anti-tubulin staining is detected at the SPB. Thus, the stabilization of microtubules is dependent upon the Ciklp::β-gal fusion, and this fu-
tion needs to associate with microtubules before nocodazole treatment to confer stability. These results clearly indicate that, similar to Kar3p::β-gal, the Ciklp::β-gal fusion can stabilize microtubules.

Like Kar3p::β-gal, the Ciklp::β-gal SPB Localization Requires Functional Karlp

In pheromone-treated cells, the SPB localization of Kar3p::β-gal is dependent upon functional Karlp, a protein which is required for nuclear fusion and appears to localize to the SPB (Conde and Fink, 1976; Rose and Fink, 1987; Vallen et al., 1992a,b). In wild-type cells arrested with mating-pheromone, Kar3p::β-gal localizes to the SPB and along microtubules; however, in a karl-Δ13 cell, Kar3p::β-gal localizes along microtubules but not at the SPB.

To determine if the Ciklp::β-gal SPB localization is also dependent upon Karlp, a MATa karl-Δ13 strain containing cikl::lacZ was treated with α-factor and stained with anti-β-gal antibodies. In this strain the microtubules are very long and often appear detached from the SPB. This phenotype is similar to that described for karl-Δ13 cells containing a Kar3p::β-gal fusion (Vallen et al., 1992b). Anti-β-gal staining of karl-Δ13 cells containing Ciklp::β-gal reveals that the fusion protein accumulates along the cytoplasmic microtubule bundle; no staining is evident at the SPB (Fig. 4). Thus, in pheromone-treated cells, there are a number of similarities between Ciklp::β-gal and Kar3p::β-gal. Both fusions localize to the SPB/microtubules and stabilize microtubules, and SPB localization of both fusion proteins requires Karlp.

In Pheromone-treated Cells, Microtubule Localization of Ciklp::β-gal Is Dependent on Kar3p and Microtubule Localization of Kar3p::β-gal Is Dependent on Ciklp

Since the localization pattern and microtubule stabilizing effect of Ciklp::β-gal and Kar3p::β-gal fusions are identical, the possibility exists that the localization of Ciklp::β-gal might depend on Kar3p and/or Kar3p::β-gal localization might require Ciklp. To test the first of these possibilities, a MATa kar3Δ strain containing cikl::lacZ was constructed. These cells were arrested with α-factor and subjected to immunofluorescence analysis. In kar3Δ cells, Ciklp::β-gal staining is evident throughout the cell and appears to preferentially reside in the cytoplasm (Fig. 5). In contrast, in wild-type and ciklΔ cells Ciklp::β-gal staining is primarily along cytoplasmic microtubules (Fig. 2; Page and Snyder, 1992). Therefore, in pheromone-treated cells, the microtubule localization of Ciklp::β-gal requires Kar3p.
In pheromone-treated cells, the SPB localization of Ciklp::B-gal requires Karlp. Strains Y808, karl-A13, (A) and Y804, KAR/, (B) containing the ciM::lacZ plasmid (pB20) were arrested with s-factor and stained with anti-/ff-gal (left panel) and anti-tubulin (right panel) antibodies. In these cells the long microtubules are not connected to the SPB (the bright spots in the figure). Bar, 4.5 μm.

To evaluate the dependence of Kar3p::B-gal microtubule localization on Ciklp, immunofluorescence experiments were performed using kar3::lacZ constructs (see Materials and Methods). The kar3::lacZ fusion contains the KAR3 upstream regulatory region and the amino terminal half of the coding region; this segment encodes half of the predicted Kar3p coiled-coil domain and lacks the Kar3p putative microtubule motor domain. When wild-type MALa cells containing kar3::lacZ are treated with α-factor and stained with anti-β-gal (left panel) and anti-tubulin (right panel) antibodies, Kar3p::β-gal is detected along the cytoplasmic microtubules and diffusely in the cytoplasm and nucleus (Fig. 5). This Kar3p::β-gal pattern is identical to that detected in both wild-type and kar3A cells examined by Meluh and Rose (1990).

To determine if the Kar3p::β-gal SPB localization is dependent upon Ciklp, the localization pattern of the Kar3p::β-gal fusion in a ciklΔ strain was evaluated by staining with anti-β-gal antibodies. Similar to that observed for wild-type cells, Kar3p::β-gal localizes to the SPB in 25% of ciklΔ cells, and this localization pattern does not appear to be cell cycle specific (Fig. 6 B). Therefore, contrary to the dependence upon Ciklp for the Kar3p::β-gal microtubule localization in pheromone-treated cells, in vegetative cells the Kar3p::β-gal SPB localization appears to be independent of Ciklp. This independence is consistent with the genetic analysis described below.

We also investigated whether the localization of Ciklp::β-gal to the SPB in vegetative cells requires Kar3p. When mitotically growing wild-type cells containing the cikl::lacZ fusion are stained with anti-β-gal antibodies, 30% of the cells exhibit a detectable signal in the region of the SPB (Fig. 5; Page and Snyder, 1992). When kar3A cells with the cikl::lacZ fusion are stained with anti-β-gal, ~30% of cells exhibited strong nuclear and weak cytoplasmic staining (>1,000 cells examined; Fig. 7). Cells at different stages of the cell cycle (unbudded, small budded, and large budded cells) exhibit this staining pattern. Because the nuclear signal may obscure detection of staining at the SPB, we cannot conclusively determine whether some Ciklp::β-gal is associated with the SPB.

In Vegetative Cells, the kar3::β-gal SPB Localization Is Independent of CIKI

Vegetative wild-type cells containing the kar3::lacZ fusion were analyzed by immunofluorescence experiments. In ~25% of these cells an anti-β-gal signal is detected at the SPB (Fig. 6 A). This SPB staining is not cell cycle-dependent since the signal is detected in both unbudded, small budded and large budded cells. Occasionally weak staining of the spindle microtubules can also be seen (Fig. 6 B).

To determine if the Kar3p::β-gal SPB localization is dependent upon Ciklp, the localization pattern of the Kar3p::β-gal fusion in a ciklΔ strain was evaluated by staining with anti-β-gal antibodies. Similar to that observed for wild-type cells, Kar3p::β-gal localizes to the SPB in 25% of ciklΔ cells, and this localization pattern does not appear to be cell cycle specific (Fig. 6 B). Therefore, contrary to the dependence upon Ciklp for the Kar3p::β-gal microtubule localization in pheromone-treated cells, in vegetative cells the Kar3p::β-gal SPB localization appears to be independent of Ciklp. This independence is consistent with the genetic analysis described below.

In Vegetative Cells, ciklΔ and kar3Δ Strains Possess Similar, but not Identical, Phenotypes

Deletion of either CIKI or KAR3 results in several similar vegetative phenotypes which include elevated chromosome loss, temperature sensitivity for growth at 37°C, and defects in spindle establishment and/or maintenance (Page and Snyder, 1992; Meluh and Rose, 1990). Sporulation of a ciklΔ/CIKI strain and tetrad analysis after growth at 25°C results in four equally sized colonies, indicating that at this temperature the ciklΔ does not have a severe effect upon cell growth (>60 tetrads analyzed). However, when a isogenic kar3A/KAR3 strain is subjected to the same analysis at 25°C, a typical tetrad gives rise to two wild-type colonies and two smaller kar3Δ colonies (16 tetrads analyzed). Thus, the kar3A mutation has a greater effect upon vegetative cell growth than the ciklΔ mutation.

Tetrad analysis of a ciklΔ/CIKI kar3A/KAR3 strain reveals that ciklΔ kar3Δ cells are viable, and the colony size of the double mutant is equal to that of a CIKI kar3Δ strain. These results are consistent with Ciklp operating in the same path-
Figure 5. In pheromone-treated cells, the microtubule localization of Ciklp::β-gal and Kar3p::β-gal depend upon KAR3 and CIKI, respectively. A kar3Δ strain (Y1137-5C) containing the cik1::lacZ plasmid (pB20) (A) was treated with α-factor and subjected to immunofluorescence analysis. Wild-type strain Y431 (B) and cik1Δ strain Y1119-37D (C), each containing the kar3::lacZ plasmid (pB41), were also arrested with α-factor and treated similarly. The left panel shows cells stained with anti-β-gal; the central and right panels exhibit anti-tubulin and Hoechst 33258 staining, respectively. Bar, 4.5 μm.
Figure 6. In vegetative cells, the SPB signal of Kar3p::β-gal is independent of CIK1. Vegetatively growing wild-type (A, Y431) and ciklA (B, Y1119-3.7D) cells containing the kar3::lacZ plasmid (pB41) were stained with anti-β-gal antibodies (left panel) and anti-tubulin antibodies (right panel), respectively. Bar, 4.5 μm.

way with Kar3p. Assuming that Kar3p and Ciklp participate together in some microtubule functions, the greater effect upon cell growth of the kar3A would suggest that KAR3 functions in some microtubule processes without CIKI. This hypothesis is compatible with the independence of the Kar3p::β-gal SPB localization upon Ciklp in vegetatively growing cells.

To further evaluate the role of Ciklp in Kar3p-dependent processes, a genetic suppression test was used. CIN8 and KIP1 are thought to encode functionally redundant kinesin-related proteins (Roof et al., 1992a; Hoyt et al., 1992). Deletion of either gene is not lethal; however, a cin8Δ kiplΔ strain is inviable. A kiplΔ cin8-3 strain is temperature-sensitive for growth at 33°C. Deletion of KAR3 suppresses this phenotype such that kar3Δ kiplΔ cin8-3 cells can form colonies at 33°C (Saunders and Hoyt, 1992). Since Ciklp and Kar3p share several similarities, a ciklΔ kiplΔ cin8-3 strain was constructed to determine if deletion of CIKI could also suppress the KiplΔ Cin8- phenotype. Of the six different ciklΔ kiplΔ cin8-3 strains examined, all are temperature-sensitive for growth at 33°C. Thus, deletion of CIKI, unlike deletion of KAR3, does not suppress the KiplΔ Cin8- phenotype.

CIKI and KAR3 Interact as Determined by the Two-Hybrid System

Since the Ciklp::β-gal and Kar3p::β-gal localization patterns are similar and localization of each fusion protein is dependent upon the presence of the other wild-type protein, we postulated that Ciklp and Kar3p might interact physically or be part of the same complex. This hypothesis was tested using three different assays: the two-hybrid system, co-immunoprecipitation and localization of authentic Ciklp along Kar3p::β-gal stabilized microtubules.

The two hybrid system utilizes two different fusion plasmids, one encoding the lexA DNA binding domain (lexA0b) and the other encoding the Gal4 activation domain (Gal4ab) (Fields and Song, 1989; Golemis and Brent, 1992). If the lexA0b and the Gal4ab segments are separately fused to two proteins capable of forming a protein–protein complex, the DNA binding and transcriptional activation domain may be brought together and activate transcription of a lexA-responsive lacZ reporter construct.

To investigate the potential protein-protein interaction between Ciklp and Kar3p, the appropriate gene fusions were constructed. Two different CIKI DNA fragments were fused to sequences encoding the lexA DNA binding domain. The larger fusion contains 71% of the CIKI coding region (amino acids 20–446) and includes the entire putative coiled-coil domain. The smaller lexA::cikl fusion encodes amino acids 20–207 which includes 75% of the coiled-coil region. The GAL4::kar3 fusion encodes amino acids 20–207 which includes 75% of the coiled-coil region. The GALA::kar3 fusion encodes 70% of the KAR3 coding sequence from amino acids 12 to 515. This fusion contains the potential coiled-coil domain but lacks the microtubule-motor domain.

The combination of either lexA::cikl fusion and the GALA plasmid (without KAR3 sequences) does not elevate expression of lacZ (Table II). This result indicates that the CIKI fusions cannot activate transcription or interact with the GALA activation domain. The combination of the GALA::kar3 fusion and the lexA plasmid is also incapable of increasing lacZ transcription above that of the negative control. However, when the reporter strain contains both the lexA:: cikl(20–446) and GALA::kar3 plasmids, the expression of lacZ, as determined by β-gal activity, is increased 180-fold (Table II). This activation of transcription indicates that Ciklp and Kar3p interact to form a protein complex. In order to determine the region of Ciklp that interacts with Kar3p, the β-gal activity of a strain containing GALA::kar3 and the smaller lexA::cikl(20–207) fusion was evaluated. Although
the lexA::cikl(20–207) fusion includes less than 50% of the CIK1 present in the larger fusion, this smaller construct in combination with GAL4::kar3 elevates lacZ expression to a comparable level (210-fold). Therefore, as determined by the two hybrid system, Ciklp and Kar3p interact, and this interaction may be facilitated by a region within the amino-terminal half of CIK1.

CIK1 Co-Immunoprecipitates with KAR3

Interaction of Ciklp and Kar3p in vivo was further tested by co-immunoprecipitation experiments. Wild-type and kar3Δ strains were incubated in the presence or absence of α-factor and total cellular protein was extracted under non-denaturing conditions. Kar3p complexes were immunoprecipitated from

Table II. CIK1- and KAR3-dependent Transcriptional Activation by the Two Hybrid System

| DNA binding domain hybrid | Activation domain hybrid | Colony color | β-gal activity |
|---------------------------|--------------------------|--------------|---------------|
| LEXA(1-87)-CIK1(20-446)   | GAL4(768-881)            | White        | 0.1           |
| LEXA(1-87)-CIK1(20-446)   | GAL4(768-881)-KAR3(12-515) | Blue         | 18            |
| LEXA(1-87)-CIK1(20-207)   | GAL4(768-881)            | White        | 0.1           |
| LEXA(1-87)-CIK1(20-207)   | GAL4(768-881)-KAR3(12-515) | Blue         | 21            |
| LEXA(1-87)                | GAL4(768-881)            | White        | 0.1           |
| LEXA(1-87)                | GAL4(768-881)            | Blue         | 0.1           |
| LEXA(1-87)-GAL4(87-881)   |                          | Blue         | 188           |

Numbers adjacent to gene designations refer to codon numbers. β-gal activity is presented in arbitrary units.
Figure 8. Ciklp co-immunoprecipitates with Kar3p in α-factor-treated cells. Proteins were isolated from a wild-type strain (MS10) or kar3Δ strain (MS524) grown vegetatively (veg) or treated with α factor (αF) and immunoprecipitated with anti-Kar3p antibodies. The immunoprecipitates were analyzed by immunoblot analysis. (A) The 88-kD Kar3p (KAR3) was detected by anti-Kar3p antibodies in both vegetative and pheromone-treated wild-type cells and was not evident in kar3Δ cells. Immunoglobulin heavy chain (IgG) and additional non-specific bands (−) were detected by the secondary antibody in the absence of primary antibody. (B) Immunoblots prepared as in (A) were probed with anti-Ciklp antibodies. The 77-kD Ciklp (CIK1) co-immunoprecipitated from pheromone-treated wild-type cells, and was not detected in immunoprecipitates from vegetative cells or kar3Δ cells.

The Presence of Kar3p::β-gal Results in Altered Localization of Ciklp

In wild-type MA1a cells arrested with α-factor, the authentic Ciklp appears associated with the SPB, but is not detected as colocalizing with microtubules (Fig. 9; Page and Snyder, The Journal of Cell Biology, Volume 124, 1994).
Localization of Authentic Ciklp vs Ciklp::B-gal Fusion

Authentic Ciklp localizes to the SPB in pheromone-treated cells; whereas, the fusion protein localizes to both the SPB and microtubules. This latter observation indicates that Ciklp associates with microtubules. There are at least two possibilities for where Ciklp might function. Ciklp might interact with microtubules while attached to the SPB. Alternatively, Ciklp, could be part of a minus end motor protein complex, which accumulates at its SPB destination (Page and Snyder, 1993). The motor directionality of Kar3p is not known, but it has been hypothesized to be a minus end motor (Saunders and Hoyt, 1992). The Ciklp::B-gal fusion might be impeded in its transit and thereby allow detection of the protein along microtubules. Regardless of which model is correct, the Ciklp::B-gal fusion indicates an interaction of this protein with microtubules which is not evident by localization of the authentic protein. Thus, the use of fusion proteins, in addition to increasing sensitivity for immunodetection experiments, can help provide information about the function of a particular protein.

B-gal fusions have been extremely useful for determining the localization domain of many proteins (e.g., Silver et al., 1984; Hall et al., 1984; Trueheart et al., 1987). Analysis of Ciklp and Kar3p fusions has determined that the SPB/microtubule localization domain is within the amino terminal half of Ciklp and the amino terminal 42% of Kar3p (Page and Snyder, 1992; Meluh and Rose, 1990). In this latter case, the Kar3p::B-gal fusion is devoid of its putative motor domain, thereby allowing analysis of a separate microtubule interacting domain which requires Ciklp.

Localization of Kar3p::B-gal Requires Ciklp in Pheromone-treated Cells

In wild-type cells treated with pheromone, both Ciklp::B-gal and Kar3p::B-gal fusions localize to cytoplasmic microtubules and the SPB. In contrast, in kar3A cells treated with pheromone, the Ciklp::B-gal fusion localizes throughout the cell, with a majority of it in the cytoplasm; it does not localize to microtubules. The Kar3p::B-gal fusion does not localize to microtubules or the SPB in pheromone-treated ciklΔ cells but instead, appears to be largely present within the nucleus. These results indicate that Ciklp and the nonmotor domain of Kar3p depend upon one another for localization to microtubule-associated structures. Consistent with this hypothesis, loss of Ciklp function yields defects in karyogamy and chromosome segregation that are similar to those of kar3Δ defects. Formation of this Ciklp-Kar3p complex and its association with microtubules/SPB are likely to be important for mediating the activities of the putative Kar3p motor. This is the first example of regulated localization of a microtubule motor protein.

The data presented above also suggest that nuclear-cytoplasmic compartmentalization of both Ciklp and Kar3p is regulated by pheromone treatment. In a kar3Δ strain, Ciklp::B-gal is detected in the nucleus during vegetative growth, but most of it is evident in the cytoplasm after α-fac-
KAR3 has Functions during Vegetative Growth That Do Not Require CIK1

The phenotypes of cik1Δ and kar3Δ strains during vegetative growth are very similar, but not identical (see introduction and Results for similarities). kar3Δ strains grow slower than cik1Δ strains, and kar3Δ, but not cik1Δ, can partially suppress the Cin8− Kip1Δ phenotype. Furthermore, during vegetative growth the localization of the KAR3p:β-gal fusion does not require Ciklp. Thus, we speculate that another Kar3p interacting protein is present in vegetatively growing cells and that this protein has a function related to Ciklp. The Ciklp redundant function is unlikely to be very important for karyogamy, because cik1Δ cells exhibit the same severe defects in intercellular karyogamy assays as kar3Δ cells. Redundancy for putative kinesin heavy chains have been previously described for KIP1 and CIN8 (Roof et al., 1992a; Hoyt et al., 1992); thus, it is possible that kinesin heavy chain interacting proteins (and light chains) are also redundant.

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