Direct Interaction between Yeast Spindle Pole Body Components: Karlp Is Required for Cdc31p Localization to the Spindle Pole Body

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Abstract. The Saccharomyces cerevisiae genes KAR1 and CDC31 are required for the initial stages of spindle pole body (SPB) duplication in yeast. The Cdc31 protein is most related to caltractin/centrin, a calcium-binding protein present in microtubule organizing centers in many organisms. Because of a variety of genetic interactions between CDC31 and KAR1 (Vallen, E. A., W. Ho, M. Winey, and M. D. Rose. 1994. Genetics. In press), we wanted to determine whether Cdc31p and Karlp physically interact. Cdc31p was expressed and purified from Escherichia coli and active for binding calcium. Using a protein blotting technique, Cdc31p bound to Karlp in vitro via an essential domain in Karlp required for SPB duplication (Vallen, E. A., M. A. Hiller, T. Y. Scherson, and M. D. Rose. 1992a. J. Cell Biol. 117:1277-1287). By immunofluorescence microscopy, we determined that the interaction also occurs in vivo. Cdc31p was localized to the SPB in wild-type cells but was mislocalized in a karl mutant strain. In a karl mutant containing a dominant CDC31 suppressor, Cdc31p was again localized to the SPB. Furthermore, the localization of Cdc31p to the SPB was affected by the overexpression of Karlp-β-galactosidase hybrids. Based on these data, we propose that the essential function of Karlp is to localize Cdc31p to the SPB, and that this interaction is normally required for SPB duplication.
fore, localization to the SPB probably reflects the essential role of KARI in SPB duplication.

Mutations in KARI result in phenotypes very similar to those produced by mutations in the CDC31 gene. Both karl and cdc31 mutations block SPB duplication and the mutants arrest as large budded cells with increased ploidy (Rose and Fink, 1987; Schild et al., 1981). Both mutations block very early in SPB duplication, resulting in a single, abnormally enlarged SPB lacking the associated half-bridge and satellite structures (Byers, 1981; Rose and Fink, 1987). Cdc3lp is a member of the calmodulin family of calcium-binding proteins (Baum et al., 1986) and shares greatest homology to the protein caltractin/centrin from Chlamydomonas reinhardtii (Huang et al., 1988b; Salisbury et al., 1988). Caltractin was identified as a major calcium-binding protein in the basal body (Huang et al., 1988a) which serves as the microtubule organizing center in Chlamydomonas. Because mutations in centrin/caltractin are defective for basal body localization and/or segregation (Taitton et al., 1992), it is likely that this centrosomal component has a conserved function among diverse organisms. A human homologue of caltractin was identified in the centrosome of each cell type studied (Lee and Huang, 1993), providing further support for the fundamental role of this protein. Based on its homology to caltractin and its mutant phenotype, Cdc31p was predicted to be a SPB component. This was recently confirmed by immunoelectron microscopy, which determined that Cdc31p is a component of the half-bridge of the SPB (Spang et al., 1993).

The MPSI and MPS2 genes were identified by screening temperature sensitive mutants for monopolar spindle formation (Winey et al., 1991). The mps-l mutation blocks SPB duplication before satellite formation with an enlarged half-bridge structure that is lacking in karl and cdc31 mutants. Order of function experiments have led to the suggestion that MPSI acts downstream of CDC31. The mps-1 mutation does not block SPB duplication but produces a defective SPB that lacks the inner plaque. Consequently, mps2 mutants are defective for nuclear microtubule attachment. Mutations in the NDC1 gene produce a phenotype that is indistinguishable by electron microscopy from the mps-2 mutation (Thomas and Botstein, 1986; Winey et al., 1993). The Ndc1 protein localizes to the nuclear envelope and may be involved in inserting the new SPB into the envelope (Winey et al., 1993). Although the MPS genes are clearly required for SPB function, it is not yet known whether they encode SPB components.

Several SPB components have been identified by a biochemical approach. A partially purified yeast SPB preparation was used to generate a pool of monoclonal antibodies (Rout and Kilmartin, 1990). Several of the antibodies specifically recognize discrete structures of the SPB. A 90-kD protein localizes to the inner and outer plaques, and a 110-kD protein (Spcl100) localizes to the nuclear region of the central plaque. SPCl10 is identical to NUPl and has a predicted coiled-coil structure (Kilmartin et al., 1993; Mizuyan et al., 1992). The protein regulates the spacing between the central plaque and the ends of microtubules. It was recently shown that Spcl100 is the essential mitotic target of calmodulin, which is also a SPB component, but the function of this interaction remains unclear (Geiser et al., 1993).

Despite the identification of genes and SPB components that may be involved in SPB duplication, the details of the SPB duplication process remain obscure. In particular, the number of SPB components is not known, nor how they physically interact to assemble a new SPB. One approach to this problem is the use of genetic techniques to identify interacting gene products. The karlAl7 allele causes a temperature-sensitive defect in SPB duplication because of a small deletion within the SPB domain (Vallen et al., 1992a). Dominant CDC31 suppressor mutations and high copy wild-type CDC31 were isolated as suppressors of karlAl7 (Vallen et al., 1994), suggesting that CDC31 and KARI interact.

All nine spontaneous CDC31 suppressors of karl map to a COOH-terminal region of Cdc31p, suggesting that this domain is critical to the mechanism of karl suppression. Since the strongest CDC31 suppressors suppress a complete deletion of KARI, the mechanism of suppression cannot result from altered interaction between the two proteins. Some type of interaction between the CDC31 suppressors and karl must remain during suppression, however, because the stronger suppressors become supersensitive to slight increases in wild-type KARI dosage. In the strongest suppressor, even a single extra copy of the KARI gene was found to be toxic. We were, therefore, led to test whether Karlp and Cdc31p physically interact by direct means.

In this paper, we show that Cdc31p directly interacts with the Karl protein. As predicted by the genetic results, Cdc31p interacts specifically with the SPB domain of Karlp. In addition, we show that Cdc31p localizes to the SPB in intact cells and that this localization depends on KARI. We propose that in wild-type cells Karlp localizes Cdc31p to the SPB as an essential step leading to SPB duplication.

Materials and Methods

Strains, Microbial Techniques, and Plasmid Constructions

All yeast strains used are listed in Table I. Yeast media and microbial techniques were as described by Rose et al., 1990. All enzymes were obtained from New England Biolabs Inc. (Beverly, MA) and were used according to the supplier specifications. Linkers were synthesized at Princeton University.

To construct plasmid pMR2298, which contains the full-length CDC31 gene fused to the T7 promoter, an NdeI site was introduced at the initial methionine of CDC31. Site-directed mutagenesis was performed on CDC31 to change the sequence from AGT ATG to CAT ATG, generating an NdeI site. The mutagenesis was performed using the Altered Sites Kit from Promega Corp. (Madison, WI) according to the manufacturer's directions.

The mutagenized gene was digested with SalII and filled in to create a blunt site at the 3' end of the CDC31 gene. It was then digested with NdeI. The resulting 1.95-kb NdeI/blunt CDC31 fragment was ligated into the puc3a vector (Studier et al., 1990) that had been digested with BamHII, filled in, and then digested with NdeI.

Cdc31p Purification

To induce Cdc31p expression in Escherichia coli, a saturated overnight culture of strain MR2303, which contains full-length CDC31 fused to the T7 promoter, was grown in Luria broth-ampicillin (25 µg/ml) and diluted 100-fold into 1 l of fresh Luria broth-ampicillin media. After 2 h of growth at 37°C, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The induced culture was grown for an additional 2 h at 37°C and then harvested at 4°C in a rotor at 10 krpm for 5 min (J14; Beckman Instruments). The pellet was resuspended in 4 ml of 2X native sample buffer (100 mM Tris-Cl, pH 8.0, 0.2% bromophenol blue, and 20% glycerol) containing 5 mM CaCl2 and a cocktail of protease inhibitors (chymostatin, leupeptin, aproatin, pepstatin A, and 4-(2-aminomethyl)-ben-
Spindle Pole Body Components Karlp and Cdc31p Interact

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Table 1. Strains Used

| Strain          | Genotype                              | Source |
|-----------------|---------------------------------------|--------|
| MS10            | MATa ura3-52 leu2-3 leu2-112 ade2-101  | *      |
| MY424           | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR404 | †      |
| MY902           | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR406 | †      |
| MY906           | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR448 | †      |
| MY967           | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR792 | †      |
| MY1499          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR1850 | †      |
| MY2908          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2345 | †      |
| MS2082          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2223 | †      |
| MS2623          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2223 | †      |
| MS2626          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2345 | †      |
| MS3027          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2345 | †      |
| MS3148          | MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAl1, pMR2345 | †      |

* All strains designated MS are isogenic with strain S288 C.
† Vellen et al. (1992b).
‡ Vellen et al. (1992a).

genesulonfluoride, HCl (AEBSF) at 2 μg/ml each final concentration). AEBSF was obtained from CalBiochem Corp. (La Jolla, CA) and all other protease inhibitors were obtained from Sigma Immunocutemns (St. Louis, MO). The suspension was lysed by five bursts of sonication for 45 s with 30 s intervals on ice between each round. The insoluble material was pelleted at 4°C in a rotor at 10 K rpm for 15 min (20; Beckman Instruments). The supernatant was run on a 12% nondenaturing polyacrylamide gel at 175 V.

Cdc31p was purified by electrophoresis of the protein from the gel. A strip of the gel was soaked in 0.19 M Tris, 0.1% SDS for 10 min followed by staining with 0.3 M CuCl2 until the bands could be visualized (>10 min) as described by Lee et al., 1987. The strip was aligned with the gel to identify Cdc31p. The protein band was then excised and eluted from the gel in native running buffer (25 mM Tris, 250 mM glycerol) for 2 h at 40°C. The protein was collected and concentrated when necessary according to manufacturer's directions (Centricon-10; Amicon, Beverly, MA).

Cdc31p Calcium Binding and Electrophoretic Shifts

Purified Cdc31p was shown to bind calcium essentially as described by Davis et al., 1986. Pure Cdc31p was separated by electrophoresis, transferred to Zeta-probe membrane (Bio-Rad Laboratories, Richmond, CA) and incubated with 45Ca at 2 μM final concentration. Cdc31p electrophoretic shifts were demonstrated by electrophoresis of bacterial extracts and pure Cdc31p in the presence of 10 mM EGTA or 10 mM calcium chloride on 15% nondenaturing gels with a 3:2 acrylamide/bisacrylamide ratio. The bacterial extracts were prepared as described above.

Cdc31p Labeling with 45Ca

5-10 μg of purified Cdc31p was dialyzed against 0.1 M borate buffer, pH 8.4, overnight at 4°C. This protein was labeled according to the specified directions with a sulfur labeling kit (Amersham Corp., Arlington Heights, IL) that adds 35S to free amino groups. The labeled protein was separated from the unincorporated label on a 20 cm × 0.8-cm Sephadex G25 (Sigma Immunocutemns) column that had been equilibrated with 0.05 M phosphate buffer (pH 7.5, 0.1 M NaCl).

Preparation of Extracts

Bacterial Karlp extracts were prepared from strain MR1578, which carries the KARp gene (lacking the last 20 amino acids) fused to the 17 promoter. A midlog culture grown at 37°C was induced with 1 mM IPTG. Extracts were prepared by harvesting 1 ml of bacteria before induction or 2 h after the addition of IPTG. The pellet was resuspended in 100 μl of 2X SDS sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 13.5% β-mercaptoethanol) and boiled for 5 min.

TrpE-Karlp hybrid protein extracts were prepared from parent strain MR308 containing plasmids pMR311 (Karlp 60-433), pMR313 (Karlp 116-433), pMR315 (Karlp 246-433), or pMR317 (Karlp 190-433). A culture of each strain was grown in M9 + cas amino acids media to saturation. 0.5 ml of this culture was diluted into 5 ml of M9, cas amino acids, ampicillin media, and grown at 30°C for 1 h. Indoleacetic acid was added (20 μl of 1 mg/ml in ethanol) and the cultures were grown for an additional 2 h at 30°C. Cells were harvested and prepared as described above.

Purified Cdc31p was shown to bind calcium essentially as described by Davis et al., 1986. Pure Cdc31p was separated by electrophoresis, transferred to Zeta-probe membrane (Bio-Rad Laboratories, Richmond, CA) and incubated with 45Ca at 2 μM final concentration. Cdc31p electrophoretic shifts were demonstrated by electrophoresis of bacterial extracts and pure Cdc31p in the presence of 10 mM EGTA or 10 mM calcium chloride on 15% nondenaturing gels with a 3:2 acrylamide/bisacrylamide ratio. The bacterial extracts were prepared as described above.
polycrylamide gel. The proteins were transferred to nitrocellulose. Cdc31p was visualized by Ponceau S staining and a strip containing Cdc31p was excised. The nitrocellulose strip was fragmented in 1 ml PBS by sonication until the membrane was in suspension. Freund's complete adjuvant (Sigma Immunologicals) was added in a 1:1 ratio, emulsified by sonication and injected subcutaneously. The initial boost was performed 3 wk later and subsequent boosts occurred once per month with 50-100 μg of soluble, purified Cdc31p (described above) emulsified in Freund's incomplete adjuvant from Sigma Immunologicals.

Antibodies were titered by Western blotting of wild-type yeast strain MS10, CDC31 overexpressing yeast strain MS3027, and bacterial Cdc31p extracts. Yeast extracts were prepared as described by Ohashi et al. (1982).

A affinity purification of the anti-Cdc31p antibodies was achieved by the use of a Cdc31p-affinity column. Approximately 2 mg of purified Cdc31p was coupled to resin using the Sulfo-link kit (Pierce Chemical Co., Rockford, IL). The protein coupling and antibody purification were according to manufacturer's specifications.

**Immunofluorescence**

Indirect immunofluorescence using formaldehyde fixation of intact cells was performed as described by Rose et al. (1990) and indirect immunofluorescence using MeOH, acetone fixation of spheroplasts was performed as described by Rout and Kilmartin (1990). Yeast strains containing pGAL-Karlp-β-galactosidase hybrid proteins (strains MY906, MY976, and MY290B) were induced by the addition of galactose to 2% for 6 h. The cells were harvested by filtration and formaldehyde was added to 4% at 23°C for 5, 10, or 15 min. For temperature shifts of yeast strains (strains MY424, MS2082, MS2523, MS2562, and MS1458), the strains were grown to 1 × 10⁷ cells/ml 23°C. The culture was split and half was shifted to 37°C for 4 h and the other half remained at 23°C. Immunofluorescence was performed on these strains by making spheroplasts that were then grown in Wickerham's media (Rout and Kilmartin, 1990). Spheroplasts were fixed to slides by submersion in MeOH at −20°C for 5 min followed by acetone at 23°C for 30 s. Affinity purified anti-Cdc31p antibodies were used at a 1:1,000 dilution. Anti-90-kd antibodies and antitubulin antibodies were used as SPB markers at a 1:500 dilution and were the generous gift of John Wickemann's media (Rout and Kilmartin, 1990). Spheroplasts were fixed to slides by submersion in MeOH at −20°C for 5 min followed by acetone at 23°C for 30 s. Affinity purified anti-Cdc31p antibodies were used at a 1:1,000 dilution. Anti-90-kd antibodies and antitubulin antibodies were used as SPB markers at a 1:500 dilution and were the generous gift of John Wickermann's media (Rout and Kilmartin, 1990). Secondary antibodies were used at a 1:1,000 dilution (Boehringer Mannheim Biochemicals, Indianapolis, IN). Secondary antibodies for detection of Cdc31p were FITC-conjugated goat anti-rabbit. Anti-90-kd secondary antibodies were rhodamine-conjugated goat anti-mouse, and tubulin secondary antibodies were rhodamine-conjugated goat anti-rat. All Cdc31p and 90-kd pictures in Fig. 6 were processed in the same exposure and contrast conditions to allow accurate comparisons between strains. 4,6-diamidino-2-phenylindole was obtained from Accurate Chemicals and Scientific Corp. (Westbury, NY).

**Results**

**Cdc31p Purification and Characterization**

As a first step toward testing for physical interaction between Karlp and Cdc31p, both proteins were separately expressed as yeast proteins (Studier et al., 1990). In the strain expressing Cdc31p, a protein of the expected molecular weight of 18 kD was present after induction (Fig. 1 A, lane 3) but not in the uninduced control (Fig. 1 A, lane 2). To purify Cdc31p, we took advantage of a behavior of calmodulin that Cdc31p shares (Davis et al., 1986); Cdc31p enters high percentage nondenaturing polyacrylamide gels under native conditions, unlike most E. coli proteins. Therefore, Cdc31p extracts were separated on 12% nondenaturing polyacrylamide gels. The band containing Cdc31 protein, which was well separated from all other E. coli proteins, was excised from the gel and the protein was electroeluted. We estimate the protein to be >95% pure by denaturing polyacrylamide gel electrophoresis (Fig. 1 A, lane 4).

Like calmodulin, Cdc31p has four potential calcium binding sites called EF hands (Krestinger, 1975; Baum et al., 1986). However, only two of the EF hands in Cdc31p are predicted to bind calcium. To determine whether the bacterial Cdc31p was active to bind calcium, different amounts of pure Cdc31 protein were immobilized to a nylon membrane and incubated with ⁴⁴Ca under conditions similar to those used for yeast calmodulin (Davis et al., 1986). Autoradiography of the membrane (Fig. 1 B) determined that the bacterial Cdc31p binds calcium in a manner proportional to the amount of protein present: 1, 0.3, and 0.1 μg Cdc31p. (C) Affinity-purified Cdc31p antibodies recognize yeast Cdc31p exclusively. Polyclonal antibodies against bacterial Cdc31p were generated and affinity purified. Western blot on total yeast extract (strain MS3027) was performed to show the purification of the antibodies. (Lane 1) unpurified antibodies, (lane 2) affinity-purified antibodies.

**Cdc31p Physically Interacts with Karlp**

To examine the interaction between Karlp and Cdc31p, pure Cdc31p was radiolabeled with ³⁵S and used to probe a membrane containing immobilized Karlp. The Karlp protein...
Cdc31p Interacts with the SPB Domain of Karlp

It was previously determined that Karlp has at least two separate domains that mediate its karyogamy and mitotic functions (Vallen et al., 1992a). While the intact Karlp protein is 433 residues, residues 118–191 are required for karyogamy and residues 190–260 are required for SPB duplication. Because CDC31 mutations were isolated as suppressors of a deletion of a portion of the SPB domain of karlpΔ17 (residues 191–246), we wanted to determine if the SPB domain in Karlp was necessary and/or sufficient for interaction with Cdc31p. To delineate the domain of Karlp that binds to Cdc31p, hybrid proteins containing various amino or carboxyl portions of Karlp were used to test for interaction.

A series of hybrids containing E. coli TrpE protein fused to various portions of the COOH-terminus of Karlp were expressed in E. coli (Studier et al., 1990), and denaturing polyacrylamide gel electrophoresis demonstrated that a protein of 50 kD was induced (Fig. 2 A, lane 2). After transfer to nitrocellulose, the blot was incubated with 35S-Cdc31p to test for binding. Radiolabeled Cdc31p bound to a single band in the induced cultures but not other bacterial protein present in the extracts under these conditions (Fig. 2 B, lane 2). The molecular weight of the labeled band and its presence only in induced extracts identified this protein as Karlp. Therefore, Cdc31p protein bound to Karlp.

Given that the method of labeling Cdc31p added sulphur groups, it was possible that Karlp bound because of the modifications of Cdc31p. Therefore, we used competition experiments with unlabeled Cdc31p, BSA, and bovine calmodulin to check that unlabeled Cdc31p binds Karlp. We used BSA because it is unrelated to Cdc31p and calmodulin because it is a homologue. Vertebrate calmodulin expressed in yeast has been shown to function in place of the yeast calmodulin (Davis and Thorner, 1989).

Increasing concentrations of unlabeled Cdc31p, BSA, or calmodulin were mixed with a fixed amount (0.01 μg) of radiolabeled Cdc31p and used to probe blots containing bacterial Karlp extracts. When increasing amounts of unlabeled Cdc31p were included (Fig. 3 A), binding of labeled Cdc31p was significantly decreased. However, increasing quantities of BSA (Fig. 3 B) or calmodulin (Fig. 3 C) did not decrease the binding of labeled Cdc31p. Therefore, the interaction between Cdc31p and Karlp is specific and not caused by the sulfation of Cdc31p.

Since Cdc31p binds calcium, we tested whether binding to Karlp required calcium. Membranes with immobilized Karlp were probed with Cdc31p in the presence of either 10 mM EGTA or 10 mM calcium chloride. Autoradiography of the membrane showed that Cdc31p bound Karlp in either condition, although there was an increase in background binding when EGTA was present during the incubation (data not shown). Additionally, when Cdc31p is bound to Karlp in the presence of calcium, the binding was not competed away by washes in buffer containing EGTA (data not shown). Therefore, calcium does not appear to be absolutely required for Cdc31p binding to Karlp.
Figure 4. Mapping of the Cdc31p-interacting domain. Data from NH2- and COOH-terminal fusions are summarized. Karl hybrid proteins were expressed from the designated plasmids either in yeast (Vallen et al., 1992b) or in E. coli. Electrophoretically separated proteins were blotted to membrane and probed with 35S-Cdc31p as described in Fig. 2. Taken together, these data localize the Cdc31p-binding region to residues 187-299. Delineation of the karyogamy and SPB domains is described in Vallen et al. (1992b).

To further map the residues required for binding, a series of NH2-terminal hybrids containing Karlp fused to beta-galactosidase were also probed for binding to Cdc31p. These hybrids were expressed in yeast from the inducible GAL promoter. Because the NH2-terminal hybrids rely on KARI translation initiation sequences, much less protein is made from these hybrids than from COOH-terminal hybrids, which use GALI translation initiation sequences. Therefore, the hybrids were concentrated by immunoprecipitation with anti-beta-galactosidase antibodies. After confirming that the hybrids were immunoprecipitated by Western blotting using anti-beta-galactosidase antibodies (data not shown), the immunoprecipitated proteins were probed with labeled Cdc31p. Autoradiography determined that only the longest NH2-terminal Karlp-beta-galactosidase hybrid protein, containing Karlp residues 1-299, was able to bind Cdc31p (data not shown). Hybrids that contain Karlp residues 1-58, 1-190, 1-259, and 187-259 did not appear to bind to Cdc31p. Therefore, the maximal COOH-terminal requirement for binding extends to Karlp residue 299. Taking the NH2-terminal and COOH-terminal hybrid data together mapped the Karlp domain required for Cdc31p binding to comprise residues 190-299 (Fig. 4). The Karlp SPB domain was previously shown to consist of residues 190-260. Therefore, the maximal domain of Karlp required to interact with Cdc31p in vitro corresponds to the SPB domain plus an additional 40 amino acids.

**Cdc31p Colocalizes with Karlp In Vivo**

Because the in vitro binding experiments and the genetic
data detected an interaction between Karlp and Cdc3lp, we
determined whether Karlp and Cdc3lp colocalize in vivo. It
was previously shown by immunofluorescence and electron
microscopy that Karlp-β-galactosidase hybrid proteins
localize to the SPB (Vallen et al., 1992b). Before the results
of Spang et al. (1993), we had predicted that Cdc3lp would
localize to the SPB because of the interaction with Karlp and
the homology of Cdc3lp to another known centrosomal
component.

To localize Cdc3lp, rabbit polyclonal antibodies were
generated against pure bacterial Cdc3lp. These antibodies
recognize Cdc3lp and many additional proteins in yeast as
shown by Western blotting (Fig. 1 C, lane 1). We confirmed
that the band of the predicted Cdc3lp molecular weight rec-
ognized by the antibodies was Cdc3lp because the intensity
increased when Cdc3lp was overexpressed (data not shown).
In addition, a band of the same molecular weight was ob-
erved when Cdc3lp was expressed in bacteria (data not
shown). The antibodies were affinity purified on a Cdc3lp
affinity column, and Western blotting confirmed that the
purified antibodies recognize only Cdc3lp in a yeast extract
(Fig. 1 C, lane 2).

The affinity-purified anti-Cdc3lp antibodies were used for
indirect immunofluorescence on wild-type intact yeast cells.
Cells that were fixed with formaldehyde showed only diffuse
staining of the cytoplasm (Fig. 5 E). We predicted that since
Cdc3lp and Karlp interact, increasing the concentration of
Karlp at the SPB should cause Cdc3lp to concentrate at the
SPB. Therefore, indirect immunofluorescence was performed
on yeast cells overexpressing a Karlp-β-galactosidase hybrid
protein that localizes to the SPB. In these cells, Cdc3lp shows
diffuse staining, as well as a bright dot (Fig. 5 B). The signal
was very sensitive to fixation conditions, and short
fixation was required to see the Cdc3lp localization. Under
these conditions, tubulin staining is very faint because of the
short fixation times (increasing the fixation time increased
the tubulin staining, data not shown). Nevertheless, these
dots were likely to represent the SPB because they colocal-
ized with the residual tubulin staining (Fig. 5 C) on the edge
of the nuclear envelope in some nuclei (shown by DAPI in
Fig. 5 A). Similar results to these were obtained using both
overexpressed wild-type Karlp, which mislocalizes to a perinuclear aggregate (Rose, M. D., unpublished observations),
and an overexpressed Karlp-β-galactosidase hybrid
that contained residues 187-246 (data not shown). In each
case, Cdc3lp was observed to be associated with the aggrega-
ting component of Karlp. In contrast, overexpression of a
Karlp-β-galactosidase hybrid that did not contain the SPB
domain did not cause Cdc3lp to be concentrated at the SPB
(data not shown). These results suggest that Karlp and
Cdc3lp interact in vivo, since increasing the amount of Karlp
hybrid protein at the SPB caused Cdc3lp to concentrate at
the SPB. In addition, as originally seen for localization of
Karlp-β-galactosidase (Vallen et al., 1992b), only one SPB
was stained in each cell using the anti-Cdc3lp antibodies.

Since detection of Cdc3lp epitopes might have been sensi-
tive to formaldehyde, we tried an alternative method using
methanol acetone fixation of spheroplasts (Rout and Kilmart-
in, 1990). When this method was used on wild-type intact
yeast cells, antibody to Cdc3lp stained one or two dots on
the edge of the nucleus (Fig. 6 B). These dots were shown
to be the SPB by colocalization with the anti-90-kD staining
(Fig. 6 A) on the edge of the nucleus (shown by DAPI in Fig.
6 C). The 90-kD protein is a component of the SPB that was
identified by a monoclonal antibody generated against par-
tially purified SPBs (Rout and Kilmartin, 1990). The dots
seen by Cdc3lp staining did not result from bleed-through of
the anti-90-kD signal (Fig. 6 D) or cross-reaction of the
secondary antibodies because there was no staining if the
Cdc3lp primary antibody was not added (Fig. 6 E). There-
fore, Cdc3lp is localized to the SPB in intact wild-type cells,
but was not detected by our antibodies if formaldehyde
fixation was used. Our results localizing Cdc3lp to the SPB in
intact yeast cells are consistent with the results of Spang et
al. (1993), which showed Cdc3lp localization on isolated
nuclei.

Because Cdc3lp interacts with the SPB domain of Karlp,
we determined whether Cdc3lp was properly localized to the
SPB in karl mutants. The karlΔ17 allele is a temperature-
sensitive mutation within the SPB region that certain CDC31
alleles suppress (Vallen et al., 1994). Therefore, the localiza-
tion of Cdc3lp was determined by indirect immunofluores-
cence in karlΔ17 at the permissive (23°C) and nonpermissive
(37°C) temperatures. The SPB is defined in all of the
following experiments as the dot of anti-90-kD staining (first
column of Fig. 6) on the edge of the nucleus (shown by DAPI
in the last column of Fig. 6). At the permissive temperature,
Cdc3lp localized to the SPB, although there seemed to be an
increase in the cytoplasmic staining (data not shown). At the
nonpermissive temperature, Cdc3lp was not localized to the
SPB; instead, there was diffuse and punctate cytoplasmic...
staining of Cdc3lp (Fig. 6 H). The mislocalization was not an effect of the higher temperature because Cdc3lp still localized to the SPB in wild-type cells at 37°C (data not shown). Therefore, the mutation in the Karlp SPB domain affected the localization of Cdc3lp, confirming an interaction between these two proteins in vivo and in vitro.

Since wild-type Cdc3lp did not localize to the SPB at the nonpermissive temperature in karlΔ17, we tested whether a CDC31 suppressor mutation restored localization to the SPB at the nonpermissive temperature. Accordingly, immunofluorescence microscopy was performed on a karlΔ17, CDC31-16 suppressor strain at 37°C. Strikingly, Cdc31-16p was localized to the SPB in the suppressor strain (Fig. 6 K). In addition, Cdc31-16p was also localized to the SPB in a strain containing a complete deletion of the KARI gene (karlΔ2) (Fig. 6 N). Therefore, at least one of the CDC31 suppressor proteins was localized to the SPB independently of KARI function. Since the CDC31-16 mutation causes temperature-sensitive growth in a KARI+ background (Vallen et al., 1994), we also determined the localization of Cdc3lp in this strain at the nonpermissive temperature. Again, Cdc3lp was able to localize to the SPB (Fig. 6 Q). Therefore, the specific temperature-sensitive defect of CDC31-16 is different from that conferred by karlΔ17, which does not localize Cdc3lp, although both lead to a block in SPB duplication.

Discussion

KARI and CDC31 Physically Interact

We present evidence that Cdc3lp physically interacts with Karlp in agreement with previous genetic data (Vallen et al., 1994). Cdc3lp was expressed and purified from E. coli and the bacterial Cdc3lp was shown to be an active protein by its ability to bind 45Ca and undergo calcium-dependent shifts in electrophoretic mobility. Cdc3lp was found to bind to Karlp in a gel blot overlay system in which soluble 35S-Cdc3lp was allowed to bind to Karlp in a gel blot overlay system in which soluble 35S-Cdc3lp was allowed to bind to immobilized Karlp. The interaction between Cdc3lp and Karlp was observed between both proteins when Karlp was expressed in bacteria or in yeast. Therefore, posttranslational modifications that might occur in yeast were not required to detect binding in vitro, although they may play an important role in vivo. The inter-

Figure 6. Immunofluorescent localization of Cdc3lp in wild-type and mutant cells. Affinity-purified Cdc3lp antibodies were used for indirect immunofluorescence on spheroplasts that were MeOH, acetone fixed. Cdc3lp localizes to the SPB (B) in a wild-type strain (strain MY424) as defined by anti-90-kD staining (A) on the edge of the nucleus (DAPI, C). Cdc3lp staining is not caused by bleed-through of the anti-90-kD staining (D) because there is no Cdc3lp staining when Cdc3lp primary antibody is not added (E). In a karlΔ17 mutant at 37°C, Cdc3lp is no longer localized to the SPB (H). Cdc3lp is relocalized to the SPB in karlΔ17 at 37°C when the CDC31-16 suppressor is present (K). Cdc3lp also localizes to the SPB when CDC31-16 is present in a complete karl deletion strain (N) as well as in a KARI+ background (Q). A, D, G, J, M, and P define the SPBs by anti-90-kD staining of the edge of the nucleus (DAPI) staining in C, F, I, L, N, and R. Cdc3lp staining is shown in panels B, E, H, K, N, and Q. G–I are strain MS2082, J–L are strain MS3148, M–O are strain MS2626, and panels P–R are strain MS2623.
action is specific because binding can be competed by increasing amounts of unlabeled Cdc3lp but not by BSA. In addition, the interaction is more specific for Cdc3lp than other calmodulin homologues because bovine calmodulin did not compete away binding at the concentrations tested. No major bacterial proteins were seen to bind Cdc3lp, but there are additional Cdc3lp-binding proteins in yeast that may be potential Cdc3lp targets. Although the interaction between Cdc3lp and Karlp did not appear to absolutely require calcium, we cannot exclude the possibility that labeling of Cdc3lp caused the protein to adopt a conformation similar to the calcium-bound state or that the binding constant for Karlp or calcium is significantly altered.

We have shown that the interaction of Cdc3lp with Karlp requires the previously identified SPB domain of Karlp. Although Cdc3lp shares homology with calmodulin, the Karlp SPB domain is not similar to any known calmodulin-binding domain. The SPB domain of Karlp is essential for the mitotic function of KARI (Vallen et al., 1992a), as well as being both necessary and sufficient for the localization of Karlp-β-galactosidase hybrids to the SPB (Vallen et al., 1992b). By these in vivo criteria, the SPB domain was defined as being composed of residues 190–260. In contrast, gel blot overlay assays on Karlp hybrids determined that residues 190–299 are required in vitro for Cdc3lp binding, and similar results were obtained for equivalent hybrids expressed in yeast or E. coli. However, we also showed that overexpression of a Karlp-β-galactosidase hybrid containing only residues 187–246 was sufficient to concentrate Cdc3lp at the SPB in vivo. It is likely the requirement for a slightly larger region for binding in vitro reflects either the requirement for refolding of Karlp in vitro or the stabilization of a Karlp-Cdc3lp complex in vivo by other protein interactions.

Although wild-type Cdc3lp could not be detected at the SPB by indirect immunofluorescence if formaldehyde fixation was used, overexpression of Karlp-β-galactosidase hybrids that localize to the SPB likewise caused Cdc3lp to be concentrated at the SPB. Under these conditions, the detection of Cdc3lp at the SPB was dependent on the overexpression of Karlp hybrids containing the SPB domain. Furthermore, earlier experiments demonstrated that the localization of Karlp-β-galactosidase hybrids to the SPB was dependent on Cdc3lp (Vallen et al., 1992b). It seems likely that the additional Cdc3lp observed at the SPB when Karlp hybrids were overexpressed was derived from the diffusely staining cytoplasmic material. However, we cannot exclude the possibility that the presence of the hybrid has simply made the Cdc3lp at the SPB more accessible to the antibody. Nevertheless, these data show that an interaction between Karlp and Cdc3lp occurs in vivo via the SPB domain of Karlp. In addition, we detected Cdc3lp localization to the SPB in wild-type whole yeast cells if spheroplasts were fixed with methanol acetone instead of formaldehyde. Under these conditions, much of the cytoplasm is extracted and little of the cytoplasmic staining of Cdc3lp is observed. It is likely that these fixation conditions make Cdc3lp more accessible to the antibody. While this manuscript was in preparation, Cdc3lp localization to the SPB on isolated nuclei was reported using indirect immunofluorescence and immunoelectron microscopy (Spang et al., 1993). Their data suggested that Cdc3lp localizes to the half-bridge, consistent with data showing a morphological change or loss of the half-bridge in cdc31 and karl mutants (Byers, 1981; Rose and Fink, 1987). Spang et al. (1993) suggested that Cdc3lp and Karlp could not physically interact because of subtle differences in ultrastructural localization between Cdc3lp and the protein aggregate including a Karlp-β-galactosidase hybrid protein. However, we have shown that Cdc3lp and Karlp do interact and propose that the localization of the overexpressed Karlp-β-galactosidase protein may not reflect the exact localization of wild-type Karlp. Furthermore, we found that Cdc3lp was no longer localized to the SPB in a karl mutant that had a portion of the SPB domain deleted. This is additional in vivo evidence for an interaction between Karlp and Cdc3lp, and it provides a suggestion for the normal in vivo function of Karlp.

Models for Karlp and Cdc3lp Interaction

The SPB domain of Karlp was previously shown to be essential for SPB duplication. Because this domain interacts with Cdc3lp and suppressors of a mutation in this region map to CDC31, we believe that the function for Karlp in the SPB duplication pathway is to bind Cdc3lp. We propose that the temperature sensitivity of karlΔ17 is caused by a decreased interaction with Cdc3lp at the nonpermissive temperature. This idea is supported by immunofluorescence experiments that showed that Cdc3lp was mislocalized in the karlΔ17 mutant. When a dominant CDC31 suppressor allele was present, Cdc3lp was relocalized to the SPB in karlΔ17. Cdc3lp-16p was also localized to the SPB in a strain containing a complete deletion of KARI. Therefore, it seems likely that Cdc3lp needs to be localized to the SPB to carry out its essential function in SPB duplication. We propose that Karlp localizes Cdc3lp to the SPB in wild-type cells, and the decreased interaction with Cdc3lp in karlΔ17 caused Cdc3lp to be mislocalized. The mechanism of CDC31 suppression appears to involve the relocation of Cdc3lp to the SPB in the karl mutants. Given that the CDC31-16, karlΔ2 strain grows normally, it would seem that KARI's only role in mitosis is to localize Cdc3lp.

To explain the relocation of the Cdc3lp suppressors to the SPB, we propose that they have an increased affinity for another SPB component, since all the mutants have wild-type levels of Cdc31 protein (data not shown). The stronger CDC31 suppressors that suppress a complete KARI deletion (Vallen et al., 1994) would have a sufficiently increased interaction with another SPB component so that they no longer require Karlp to localize them to the SPB. In these strains, increased doses of wild-type KARI may be toxic (Vallen et al., 1994) because excess Cdc3lp is localized to the SPB, which could alter subunit stoichiometry enough to interfere with SPB assembly. Weaker CDC31 suppressors that do not suppress a complete deletion of KARI might still require KARI function, as well as increased interaction with another SPB component to localize Cdc3lp to the SPB. This idea is supported by the observation that increased levels of wild-type Cdc3lp suppress karlΔ17 (Vallen et al., 1994), possibly by stabilizing the interaction between the mutant Karlp and Cdc3lp. However, it remains possible that the weaker suppressors act via an altered interaction with the mutant karlΔ17 protein.

Our data and the results of Spang et al. (1993) have shown that Cdc3lp localizes to both SPBs in wild-type cells.
ever, previous data showed that KARl-β-galactosidase hybrid localize exclusively to the newly assembled SPB (Vallen et al., 1992b). Although this seems paradoxical, it is likely that the hybrid protein does not reflect the exact localization of wild-type KARl because it forms an aggregate containing Cdc31p and other proteins. Possibly, the KARl hybrid aggregate can only be detected at the new SPB because it is accessible to antibodies in a new SPB but not in an old SPB. Alternatively, the KARl hybrid aggregate may be lost from the old SPB because of cell cycle-regulated changes in interactions that reflect wild-type functions of KARl. For example, KARl might be required for the initiation of assembly of the Cdc31p-containing structure but not required for its maintenance. Therefore, the localization of the hybrid protein would reflect a regulated affinity of KARl for another protein, possibly Cdc31p, during assembly of a new SPB. After cell division, the hybrid proteins would be lost from the SPB because of cell cycle regulation of the interaction. It will not be possible to distinguish between these possibilities until wild-type KARl is localized.

KARI and CDC31 Act in a Common SPB Duplication Pathway

We believe that KARI and CDC31 act in a common pathway to mediate SPB duplication because the proteins physically interact and because of the similarity of their mutant phenotypes. It seems likely that the dominant gain of function CDC31 suppressors arise from a higher affinity for another SPB component. Since all of the dominant suppressor mutations mapped within the COOH terminal lobe of Cdc31p (Vallen et al., 1994), we propose that it is the COOH terminus of Cdc31p that interacts with one or more additional SPB component(s). Identification of the downstream components will be critical to understanding the role of CDC31 in SPB duplication. Potential Cdc31p-interacting proteins include other kariΔ7 suppressors, such as DSK2-1 (Vallen et al., 1994). We have recently cloned the dsk2Δ gene, and we are currently determining its function in the SPB duplication pathway.

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References

Baum, P., C. Furlong, and B. Byers. 1986. Yeast gene required for spindle pole body duplication: homology of its product with Ca2+-binding proteins. Proc. Natl. Acad. Sci. USA. 83:5512-5516.

Byers, B., and B. Goetsch. 1974. Duplication of spindle plaques and integration of the yeast cell cycle. Cold Spring Harbor Symp. Quant. Biol. 38:123–131.

Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J. Bacteriol. 124:511–523.

Byers, B. 1981. Multiple roles of the spindle pole bodies in the life cycle of Saccharomyces cerevisiae. In Molecular Genetics in yeast. Alfred Benzon Symposia 16. D. von Wettstein, A. Stiererup, M. Kleid-Brandt, and J. Pfeifer. Copenhagen, pp. 119–133.

Conde, J., and G. R. Fink. 1986. A mutant of Saccharomyces cerevisiae defective for nuclear fusion. Proc. Natl. Acad. Sci. USA. 73:3651-3655.

Davis, T. N., M. S. Urea, F. R. Maslizar, and J. Thiemer. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. Cell. 47:423-431.

Davis, T. N., and J. Thiemer. 1989. Vertebrate and yeast calmodulin, despite sequence divergence, are functionally interchangeable. Proc. Natl. Acad. Sci. USA. 86:7909–7913.

Delgado, M. A., and J. Conde. 1984. Benomyl prevents nuclear fusion in Saccharomyces cerevisiae. Mol. Gen. Genet. 193:188–189.

Geiser, J. R., H. A. Sunderberg, B. H. Chang, E. G. D. Muller, and T. N. Davis. 1993. The essential mitotic target of calmodulin is the 110-kilodallon component of the spindle pole body in Saccharomyces cerevisiae. Mol. Cell Biol. 13:7913–7924.

Huang, B., D. M. Watterson, V. D. Lee, and M. J. Schibler. 1988a. Purification and characterization of a basal body-associated Ca2+-binding protein. J. Cell Biol. 107:121–131.

Huang, B., A. Menges, and V. D. Lee. 1988b. Molecular cloning of cDNA for caltractin, a basal body-associated Ca2+-binding protein: homology in its protein sequence with calmodulin and the yeast CDC31 gene product. J. Cell Biol. 107:133–140.

Huffaker, T. C., J. H. Thomas, and D. Botstein. 1988. Diverse effects of β-tubulin mutations on microtubule formation and function. J. Cell Biol. 107:1997–2010.

Jacobs, C. W., A. E. M. Adams, P. J. Szansizio, and J. R. Pringle. 1988. Functions of microtubules in the Saccharomyces cerevisiae cell cycle. J. Cell Biol. 107:1490–1426.

Kilmartin, J. V., S. L. Dyos, D. Kershaw, and J. T. Finch. 1993. A spacer protein in the Saccharomyces cerevisiae spindle pole body whose transcript is cell cycle regulated. J. Cell Biol. 123:1175–1184.

Kochanski, R. S., and G. G. Borisy. 1994. Mode of centriole duplication and distribution. J. Cell Biol. 110:1599–1605.

Kretzinger, R. H. 1975. Hypothesis: Calcium modulated proteins contain EF hands. In Calcium Transport in Contraction and Secretion. E. Carafoli, F. Clementi, W. Drabikowski, and A. Margreth, editors. Elsevier/North Holland, Amsterdam. 469–478.

Lee, D. V., and B. Huang. 1993. Molecular cloning and centrosomal localization of human caltractin. Proc. Natl. Acad. Sci. USA. 90:11093–11094.

Lee, C. A., A. Levin, and D. Branton. 1987. Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 166:308–312.

Mirriyann, C. S. Copeland, and M. Snyder. 1992. The NUP1 gene encodes an essential coiled-coil related protein that is a potential component of the yeast nucleoskeleton. J. Cell Biol. 116:1319–1332.

Ohashi, A., J. Gibson, I. Gregor, and G. Schatz. 1982. Import of proteins into mitochondria. J. Biol. Chem. 257:13042–13047.

Rose, M. D., and G. R. Fink. 1987. KARI, a gene required for function of both basal and peripheral microtubules in yeast. Cell. 48:1047–1060.

Rose, M. D., F. Winston, and P. Heiter. 1990. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 198 pp.

Rose, M. D., S. Biggins, and L. S. Satterwhite. 1993. Unraveling the tangled nature of the yeast spindle. Trends Genet. 9:119–133.

Schild, D., H. N. Anthasawamy, and R. K. Mortimer. 1981. An endomitic effect of a cell cycle mutant of Saccharomyces cerevisiae. Genetics. 97:545–562.

Spang, A., J. Campaign, U. Fackler, M. Matzner, and E. Schiebel. 1993. The calcium-binding protein cell division cycle 31 of Saccharomyces cerevisiae is a component of the half bridge of the spindle pole body. J. Cell Biol. 120:405–416.

Stabler, F. W., A. H. Rosenburg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Methods Enzymol. 18:60–83.

Taitton, B. E. S., A. Adler, J. P. Suhann, and J. W. Jarvik. 1992. Mutational analysis of centrin: an EF-hand protein associated with three distinct contractile fibers in the basal body apparatus of Chlamydomonas. J. Cell Biol. 119:1613–1624.

Thomas, J. G., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. Cell. 44:65–76.

Vallen, E. A., M. A. Hiller, T. Y. Scherson, and M. D. Rose. 1992a. Separate domains of KARl mediate distinct functions in mitosis and nuclear fusion. J. Cell Biol. 117:1277–1287.

Vallen, E. A., T. Y. Scherson, T. Roberts, K. van Zee, and M. D. Rose. 1992b. Asymmetric mitotic segregation of the yeast spindle pole cell. Cell. 69:505–515.

Vallen, E. A., W. H. Moore, and M. D. Rose. 1994. Genetic interactions between CDC31 and KARI, two genes required for duplication of the microtubule organizing center in Saccharomyces cerevisiae. Genetics. 139:57–67.

Winey, M., L. Goetsch, P. Baun, and B. Byers. 1991. MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. J. Cell Biol. 114:745–754.

Winey, M., M. A. Hoyt, C. Chan, L. Goetsch, D. Botstein, and B. Byers. 1993. CDC11: A nuclear envelope component required for yeast spindle pole body duplication. J. Cell Biol. 122:743–752.

Winey, M., and B. Byers. 1993. Assembly and functions of the spindle pole body in budding yeast. Trends Genet. 9:300–304.