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. 1992*a*. *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.

All eukaryotic organisms contain microtubule organizing centers that mediate the assembly of the microtubule-based cytoskeleton. Like the centrosome of higher eukaryotes, the microtubule organizing center in *Saccharomyces cerevisiae*, called the spindle pole body (SPB)[1], mediates the assembly of the mitotic and meiotic spindles (for reviews see Rose et al., 1993; Winey and Byers, 1993). In addition, the SPB also assembles cytoplasmic microtubules that are essential for the process of nuclear fusion during mating (karyogamy).

The SPB is a disc-shaped trilaminar structure embedded in the nuclear envelope (Byers and Goetsch, 1974; Byers and Goetsch, 1975). The central plaque is within the plane of the membrane, whereas the inner and outer plaques face the nucleoplasm and cytoplasm, respectively. In addition, the "half-bridge" structure appears to be a distinct modification of the nuclear envelope on one side of the SPB. The inner plaque organizes the nuclear microtubules that form the mitotic and meiotic spindles, while the outer plaque organizes the cytoplasmic microtubules that are required for nuclear positioning, spindle orientation, and karyogamy (Jacobs et al., 1988; Delgado and Conde, 1984; Huffaker et al., 1988).

As is the case for centriole duplication (Kochanski and Borisy, 1990), SPB duplication appears to be a conservative process that occurs early in the cell cycle before S phase (Winey et al, 1991; Vallen et al., 1992*b*). The earliest known event in SPB duplication is the formation of a precursor "satellite" structure on the cytoplasmic face of the half-bridge. This is followed by the appearance of two morphologically identical SPBs connected by a complete "bridge" structure. The bridge splits when one SPB migrates to the opposite side of the nucleus to form a bipolar spindle, resulting in two half-bridge structures, one of which remains attached to each SPB.

Several genes required for SPB duplication have been identified by mutations that affect microtubule-mediated processes. *KAR1* was identified by its role in karyogamy (Conde and Fink, 1976), and further genetic analysis demonstrated that it is required for SPB duplication (Rose and Fink, 1987). These two functions of *KAR1* are mediated by two separate domains, the karyogamy and SPB domains (Vallen et al., 1992*a*). Because Karlp-β-galactosidase hybrid proteins localize to the SPB, it was proposed that Karlp is a component of the SPB (Vallen et al., 1992*b*). The SPB domain was found to be both necessary and sufficient for localization of Karlp-β-galactosidase hybrids to the SPB. There-

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1. *Abbreviations used in this paper*: IPTG, isopropyl β-D-thiogalactopyranoside; SPB, spindle pole body.

fore, localization to the SPB probably reflects the essential role of *KAR1* in SPB duplication.

Mutations in *KAR1* result in phenotypes very similar to those produced by mutations in the *CDC31* gene. Both *kar1* 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). Cdc31p 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., 1988*b*; Salisbury et al., 1988). Caltractin was identified as a major calcium-binding protein in the basal body (Huang et al., 1988*a*) which serves as the microtubule organizing center in *Chlamydomonas*. Because mutations in centrin/caltractin are defective for basal body localization and/or segregation (Taillon et al., 1992), it is likely that this centrosomal component has a conserved function among diverse organisms. A human homologue of caltractin that localizes to the centrosome was identified (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 *MPS1* and *MPS2* genes were identified by screening temperature sensitive mutants for monopolar spindle formation (Winey et al., 1991). The *mps1-1* mutation blocks SPB duplication before satellite formation with an enlarged half-bridge structure that is lacking in *kar1* and *cdc31* mutants. Order of function experiments have led to the suggestion that *MPS1* acts downstream of *CDC31*. The *mps2-1* 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 *mps2* 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 (Spc110p) localizes to the nuclear region of the central plaque. *SPC110* is identical to *NUF1* and has a predicted coiled-coil structure (Kilmartin et al., 1993; Mirzayan et al., 1992). The protein regulates the spacing between the central plaque and the ends of microtubules. It was recently shown that Spc110p 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 *kar1Δ17* allele causes a temperature-sensitive defect in SPB duplication because of a small deletion within the SPB domain (Vallen et al., 1992*a*). Dominant *CDC31* suppressor mutations and high copy wild-type *CDC31* were isolated as suppressors of *kar1Δ17* (Vallen et al., 1994), suggesting that *CDC31* and *KAR1* interact.

All nine spontaneous *CDC31* suppressors of *kar1* map to a COOH-terminal region of Cdc31p, suggesting that this domain is critical to the mechanism of *kar1* suppression. Since the strongest *CDC31* suppressors suppress a complete deletion of *KAR1*, the mechanism of suppression cannot result from altered interaction between the two proteins. Some type of interaction between the *CDC31* suppressors and *kar1* must remain during suppression, however, because the stronger suppressors become supersensitive to slight increases in wild-type *KAR1* dosage. In the strongest suppressor, even a single extra copy of the *KAR1* 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 *KAR1*. 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 SalI 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 pet3a vector (Studier et al., 1990) that had been digested with BamHI, 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 8.8, 0.2% bromphenol blue, and 20% glycerol) containing 5 mM $CaCl_2$ and a cocktail of protease inhibitors (chymostatin, leupeptin, aprotinin, pepstatin A, and 4-(2-aminoethyl)-ben-

Table I. 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	
MY902	MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL1+, pMR404	‡
MY906	MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL1+, pMR406	‡
MY967	MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL1+, pMR448	‡
MY1499	MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL1+, pMR792	‡
MY2908	MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL1+, pMR1850	‡
MS2082	MATα ura3-52 leu2-3 leu2-112 lys2-801 ade2-101 kar1Δ17	§
MS2623	MATa ura3-52 trp1-Δ1 lys2-801 ade2-101 CDC31-16	§
MS2626	MATα ura3-52 leu2-3 leu2-112 ade2-1-1 kar1Δ2, pMR2223	§
MS3027	MATa ura3-52 leu2-3 leu2-112 ade2-101, pMR2345	
MS3148	MATα/MATα ura3-52/ura3-52 leu2-3/leu2-3 leu2-112/leu2-112 ade2-101/ade2-101 kar1Δ17/kar1Δ17 CDC31+/CDC31-16	§

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

zenesulfonylfluoride, 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 Immunochemicals (St. Louis, MO). The suspension was lysed by five bursts of sonication for 45 s with 45 s on ice between each round. The insoluble material was pelleted at 4°C in a rotor at 10 K rpm for 15 min (J20; Beckman Instruments). The supernatant was run on a 12% nondenaturing polyacrylamide gel at 175 V.

Cdc31p was purified by electroelution 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 $CuCl_2$ 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 glycine) for 2 h at 40 mA. 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 ^{45}Ca 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 38:2 acrylamide/bisacryalmide ratio. The bacterial extracts were prepared as described above.

Cdc31p Labeling with ^{35}S

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 sulphur labeling kit (Amersham Corp., Arlington Heights, IL) that adds ^{35}S to free amino groups. The labeled protein was separated from the unincorporated label on a 20 cm × 0.8-cm Sephadex G25 (Sigma Immunochemicals) column that had been equilibrated with 0.05 M phosphate buffer (0.05 M phosphate, pH 7.5, 0.1 M NaCl).

Preparation of Extracts

Bacterial Karlp extracts were prepared from strain MR1578, which contains the *KAR1* gene (lacking the last 20 amino acids) fused to the T7 promotor. 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-Kar1 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.

Yeast Karlp-β-galactosidase amino-terminal and carboxy-terminal hybrid proteins were expressed from plasmids contained in parent strain MY424 (see Table I for strain designations). Cultures were grown to saturation in selective media containing 2% glucose and used to inoculate fresh selective media containing 2% raffinose. When cultures were 5×10^6 cells/ml, hybrid protein production was induced by the addition of galactose to 2%. Cultures were induced for 6 h and then harvested. Because the NH_2-terminal hybrid proteins are expressed at low levels, immunoprecipitations were performed to concentrate the hybrid proteins. After the 6-h galactose induction, 20 ml of culture was used for immunoprecipitation. Cells were harvested, resuspended in buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.1% Triton X-100), and lysed by vortexing with glass beads for 5 X 1 min. After centrifugation to pellet the insoluble material, the supernatant was diluted with buffer (190 mM NaCl, 6 mM EDTA, 60 mM Tris, pH 7.4, 1 mM PMSF, and 0.1% Triton X-100), and monoclonal anti-β-galactosidase antibodies from Promega Corp. (Madison, WI) were added at a 1:1,000 dilution. After incubation with the antibodies overnight at 4°C, protein A–Sepharose beads were added for 1 h at room temperature. The beads were washed 3X with buffer (150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.4). To check the precipitation, Western blots were performed using the anti-β-galactosidase antibodies followed by anti-mouse horseradish peroxidase conjugate secondary antibody (Amersham Corp.) used at a 1:3,000 dilution.

Interaction Experiments

Extracts were prepared as described above and then electrophoretically separated on denaturing polyacrylamide gels. The separated extracts were transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) that were subsequently blocked in incubation buffer (50 mM Tris-Cl, pH 7.4, 1 mM $CaCl_2$, and 150 mM NaCl) containing 5% nonfat milk for 1 h at room temperature. After blocking, the membranes were probed with a 1:200 dilution of labeled Cdc31p in incubation buffer containing 1% nonfat milk for 8–16 h at 23°C. The membranes were washed 2 X 3 min in incubation buffer and then dried for ≥30 min at room temperature. Membranes were autoradiographed at −70°C with an intensifying screen. Most of the interaction experiments were performed as described above with the exceptions of the competition experiments and the calcium/EGTA experiments. The competition experiment included 0.01, 0.1, or 1 μg of unlabeled Cdc31p, calmodulin (Sigma Immunochemicals) or BSA (Sigma Immunochemicals) during the incubation. For the calcium/EGTA experiments to determine if calcium was required for interaction, incubation buffer containing 1% BSA instead of 1% milk was used with either 10 mM EGTA or 10 mM calcium chloride.

Antibody Production and Purification

Antibodies against bacterially expressed Cdc31p were generated in two New Zealand female rabbits. Proteins from 1-liter cultures of strain MR2303 expressing Cdc31p were prepared and separated on a 12% nondenaturing

polyacrylamide 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 Immunochemicals) 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 Immunochemicals.

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).

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, MY967, and MY2908) 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, MS2623, MS2626, and MS3148), the cells were grown to 1×10^7 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 Wickermann'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 Kilmartin (MRC, Cambridge, England). 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 taken at 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 in *E. coli* (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

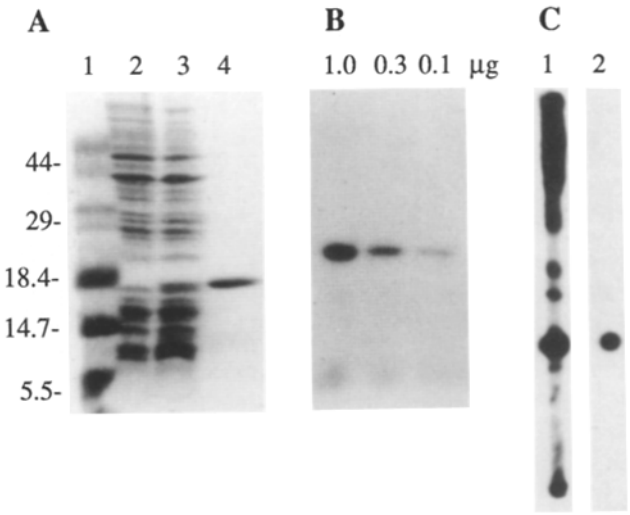


Figure 1. (*A*) Purification of Cdc31p. A bacterial extract from strain MR2303 was induced for Cdc31p expression and separated on a 15% nondenaturing polyacrylamide gel. The Cdc31p band was excised and eluted to obtain pure protein. Cdc31p was purified to homogeneity as demonstrated by electrophoresis under denaturing conditions: (Lane *1*) Prestained low molecular weight markers; (lane *2*) uninduced bacterial extract; (lane *3*) bacterial extract induced for Cdc31p expression, (lane *4*) pure Cdc31p. (*B*) Cdc31p binds ^{45}Ca. 1.0, 0.3, and 0.1 μg of pure Cdc31 protein were run on a 15% denaturing polyacrylamide gel, transferred to membrane, and incubated with ^{45}Ca. The membrane was washed and exposed to film at room temperature. Autoradiography shows that Cdc31p binds calcium in proportion 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.

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 ^{45}Ca 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.

Yeast calmodulin displays an electrophoretic shift in the presence of calcium because of a conformational change that occurs upon calcium binding (Davis et al., 1986). To determine whether the bacterial Cdc31p was active for this property, pure Cdc31p and Cdc31p bacterial extracts were separated on nondenaturing polyacrylamide gels in the presence of either EGTA or calcium chloride (data not shown). Cdc31p displayed calcium-dependent shifts in electrophoretic mobility both in pure form and in bacterial extracts, suggesting that the protein purified from bacteria was in an active conformation.

Cdc31p Physically Interacts with Karlp

To examine the interaction between Karlp and Cdc31p, pure Cdc31p was radiolabeled with ^{35}S and used to probe a membrane containing immobilized Karlp. The Karlp protein

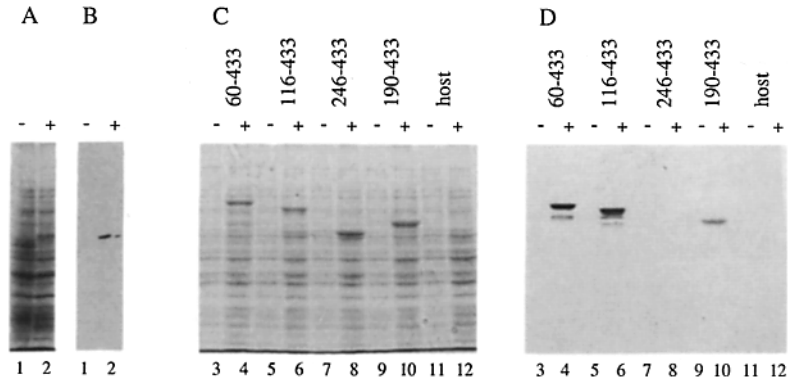


Figure 2. Cdc31p interacts with Karlp. (*A*) Uninduced (−) and induced (+) bacterial extracts expressing full-length Karlp were run on a 10% denaturing polyacrylamide gel that was Coomassie stained. (Lane *1*) Uninduced bacterial extract (lane 2) induced Karlp bacterial extract. (*B*) The proteins were transferred to nitrocellulose membrane and incubated with radiolabeled Cdc31p. The membrane was exposed to film at −70°C. The autoradiograph shows that Cdc31p binds to Karlp induced in bacteria: (lane *1*) uninduced Karlp bacterial extract; (lane 2) induced Karlp bacterial extract. (*C*) The same experiment was performed with TrpE-Karlp hybrids expressed in bacteria. Coomassie staining of a 10% denaturing polyacrylamide gel shows the induction of the hybrid proteins. The portion of Karlp present in the hybrid is listed above each lane. (*D*) Autoradiograph shows that Cdc31p binds to COOH-terminal Karlp hybrids that contain at least amino acids 190–433 (lanes *4*, *6*, and *10*), but not to Karlp hybrid that lacks amino acids 190–246 (lane *8*). Odd-numbered lanes are uninduced bacterial extracts (−), and even numbered lanes are induced (+) extracts. Hybrid 60–433 is pMR311, 116-433 is pMR313, 246–433 is pMR315, and 190–433 is pMR317. The host strain (MR308), which does not contain any Karlp hybrid, is also shown.

was 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 ^{35}S-Cdc31p to test for binding. Radiolabeled Cdc31p bound to a single band in the induced cultures but no 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, Cdc31 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.

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., 1992*a*). 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 *kar1Δ17* (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 ex-

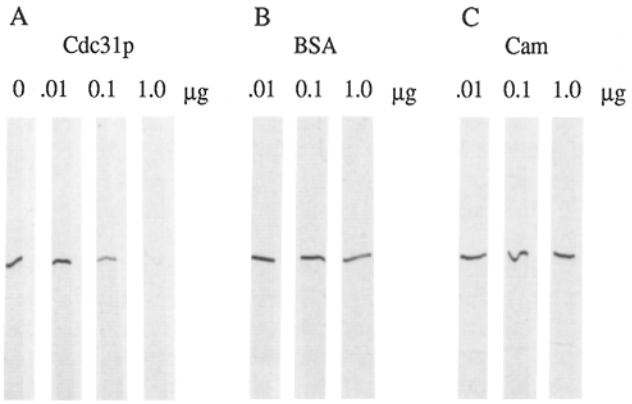


Figure 3. Cdc31p competition experiment. Radiolabeled Cdc31p (0.01 μg) was mixed with unlabeled Cdc31p (*A*), BSA (*B*), or bovine calmodulin (*C*). The amount of unlabeled Cdc31p, BSA, or calmodulin included is listed above each lane. The mixture was used to probe bacterial Karlp extracts that were separated on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose membrane. The resulting autoradiographs show that excess unlabeled Cdc31p can compete with labeled Cdc31p for binding to Karlp (*A*). However, BSA (*B*) and calmodulin (*C*) cannot compete with labeled Cdc31p.

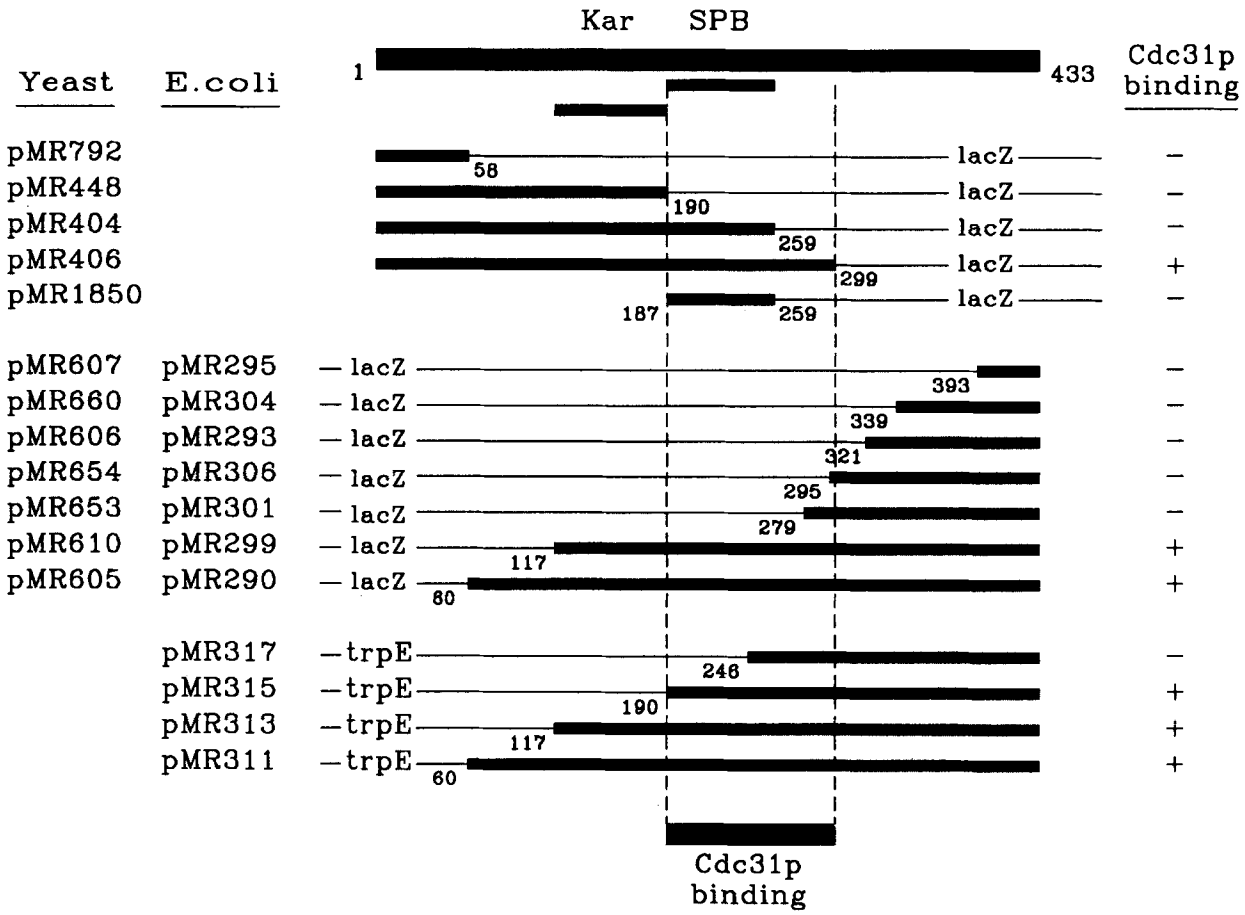


Figure 4. Mapping of the Cdc31p-interacting domain. Data from NH_2- and COOH-terminal fusions are summarized. Karl hybrid proteins were expressed from the designated plasmids either in yeast (Vallen et al., 1992*b*) or in *E. coli*. Electrophoretically separated proteins were blotted to membrane and probed with ^{35}S-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. (1992*b*).

pressed in bacteria (Fig. 2 *C*) and probed with ^{35}S-Cdc31p to map the interacting domain. As shown in Fig. 2 *D*, hybrids that contain Karlp residues 60–433 (lane *4*), 116–433 (lane *6*), and 190–433 (lane *10*) were competent to bind to Cdc31p. However, the TrpE-Karl hybrid that contained Karlp residues 246–433 (lane *8*) did not bind Cdc31p. The same result was obtained when yeast β-galactosidase-Karlp COOH-terminal hybrids containing the same portions of Karlp as the TrpE-Karlp hybrids were probed (data not shown), demonstrating that no modifications of Karlp are required for binding. Additional Cdc31p-binding proteins were present in the yeast extracts that may be targets of Cdc31p function. These data demonstrated that residues between 190–246 of Karlp are required, although not necessarily sufficient, for binding to Cdc31p. Since all of these hybrids contain Karlp residues 246–433, they do not allow a determination of the COOH terminal requirement for binding.

To further map the residues required for binding, a series of NH_2-terminal hybrids containing Karlp fused to β-galactosidase were also probed for binding to Cdc31p. These hybrids were expressed in yeast from the inducible *GAL1* promoter. Because the NH_2-terminal hybrids rely on *KAR1* translation initiation sequences, much less protein is made from these hybrids than from COOH-terminal hybrids, which use *GAL1* translation initiation sequences. Therefore, the hybrids were concentrated by immunoprecipitation with anti-β-galactosidase antibodies. After confirming that the hybrids were immunoprecipitated by Western blotting using anti-β-galactosidase antibodies (data not shown), the immunoprecipitated proteins were probed with labeled Cdc31p. Autoradiography determined that only the longest NH_2-terminal Karlp-β-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 NH_2-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 Cdc31p, we determined whether Karlp and Cdc31p 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., 1992*b*). Before the results of Spang et al. (1993), we had predicted that Cdc31p would localize to the SPB because of the interaction with Karlp and the homology of Cdc31p to another known centrosomal component.

To localize Cdc31p, rabbit polyclonal antibodies were generated against pure bacterial Cdc31p. These antibodies recognize Cdc31p and many additional proteins in yeast as shown by Western blotting (Fig. 1 *C*, lane *1*). We confirmed that the band of the predicted Cdc31p molecular weight recognized by the antibodies was Cdc31p because the intensity increased when Cdc31p was overexpressed (data not shown). In addition, a band of the same molecular weight was observed when Cdc31p was expressed in bacteria (data not shown). The antibodies were affinity purified on a Cdc31p affinity column, and Western blotting confirmed that the purified antibodies recognize only Cdc31p in a yeast extract (Fig. 1 *C*, lane *2*).

The affinity-purified anti-Cdc31 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 Cdc31p and Karlp interact, increasing the concentration of Karlp at the SPB should cause Cdc31p 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, Cdc31p 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 Cdc31p 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 colocalized 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, Cdc31p was observed to be associated with the aggregate containing Karlp. In contrast, overexpression of a Karlp-β-galactosidase hybrid that did not contain the SPB domain did not cause Cdc31p to be concentrated at the SPB (data not shown). These results suggest that Karlp and Cdc31p interact in vivo, since increasing the amount of Karlp hybrid protein at the SPB caused Cdc31p to concentrate at the SPB. In addition, as originally seen for localization of Karlp-β-galactosidase (Vallen et al., 1992*b*), only one SPB was stained in each cell using the anti-Cdc31p antibodies.

Since detection of Cdc31p epitopes might have been sensitive to formaldehyde, we tried an alternative method using methanol acetone fixation of spheroplasts (Rout and Kilmartin, 1990). When this method was used on wild-type intact yeast cells, antibody to Cdc31p 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.

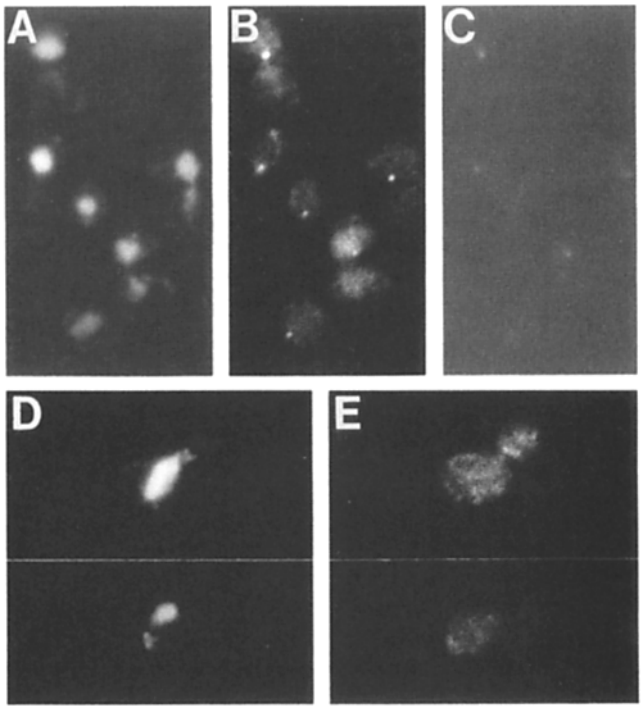
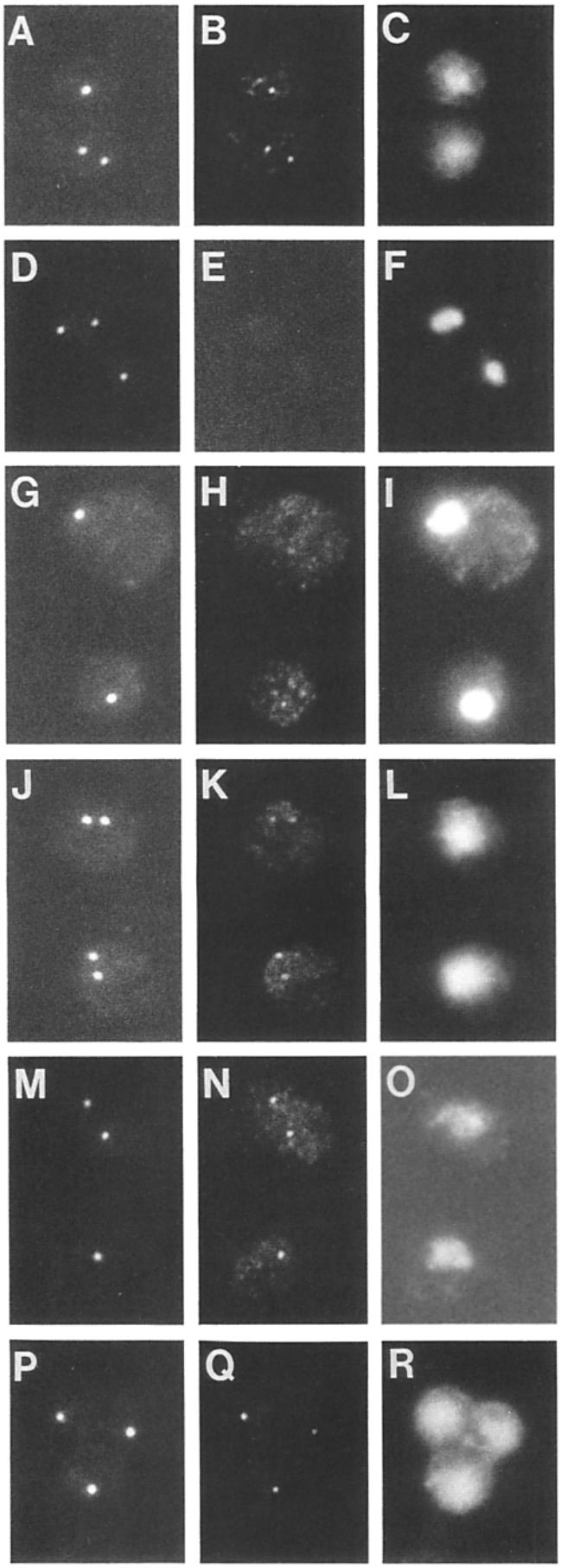


Figure 5. Immunofluorescent localization of Cdc31p using formaldehyde fixation. Affinity purified Cdc31p antibodies were used for indirect immunofluorescence on intact yeast cells (strain MY424) that were formaldehyde fixed for 15 min. DAPI staining defines the nucleus (*D*) and Cdc31p staining appears diffuse (*E*). When Cdc31p staining is performed using a strain overexpressing a Karlp-β-galactosidase hybrid protein (strain MY906), Cdc31p localizes to the SPB (*B*) as determined by the vertex of tubulin staining (*C*) on the edge of the nucleus (*A*).

6 *C*). The 90-kD protein is a component of the SPB that was identified by a monoclonal antibody generated against partially purified SPBs (Rout and Kilmartin, 1990). The dots seen by Cdc31p 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 Cdc31p primary antibody was not added (Fig. 6 *E*). Therefore, Cdc31p 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 Cdc31p to the SPB in intact yeast cells are consistent with the results of Spang et al. (1993), which showed Cdc31p localization on isolated nuclei.

Because Cdc31p interacts with the SPB domain of Karlp, we determined whether Cdc31p was properly localized to the SPB in *karl* mutants. The *karl*Δ*17* allele is a temperature-sensitive mutation within the SPB region that certain *CDC31* alleles suppress (Vallen et al., 1994). Therefore, the localization of Cdc31p was determined by indirect immunofluorescence in *karl*Δ*17* 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, Cdc31p localized to the SPB, although there seemed to be increased cytoplasmic staining (data not shown). At the nonpermissive temperature, Cdc31p was not localized to the SPB; instead, there was diffuse and punctate cytoplasmic



staining of Cdc31p (Fig. 6 *H*). The mislocalization was not an effect of the higher temperature because Cdc31p 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 Cdc31p, confirming an interaction between these two proteins in vivo and in vitro.

Since wild-type Cdc31p did not localize to the SPB at the nonpermissive temperature in *karl*Δ*17*, we tested whether a *CDC31* suppressor mutation restored localization to the SPB at the nonpermissive temperature. Accordingly, immunofluorescence microscopy was performed on a *karl*Δ*17*, *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 *KAR1* gene (*karl*-Δ2) (Fig. 6 *N*). Therefore, at least one of the *CDC31* suppressor proteins was localized to the SPB independently of *KAR1* function. Since the *CDC31-16* mutation causes temperature-sensitive growth in a *KAR1*$^+$ background (Vallen et al., 1994), we also determined the localization of Cdc31p in this strain at the nonpermissive temperature. Again, Cdc31p 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 *karl*Δ*17*, which does not localize Cdc31p, although both lead to a block in SPB duplication.

Discussion

KAR1 and CDC31 Physically Interact

We present evidence that Cdc31p physically interacts with Karlp in agreement with previous genetic data (Vallen et al., 1994). Cdc31p was expressed and purified from *E. coli* and the bacterial Cdc31p was shown to be an active protein by its ability to bind ^{45}Ca and undergo calcium-dependent shifts in electrophoretic mobility. Cdc31p was found to bind to Karlp in a gel blot overlay system in which soluble ^{35}S-Cdc31p was allowed to bind to immobilized Karlp. The interaction between Cdc31p 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 Cdc31p in wild-type and mutant cells. Affinity-purified Cdc31p antibodies were used for indirect immunofluorescence on spheroplasts that were MeOH, acetone fixed. Cdc31p 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*). Cdc31p staining is not caused by bleed-through of the anti-90-kD staining (*D*) because there is no Cdc31p staining when Cdc31p primary antibody is not added (*E*). In a *karl*Δ*17* mutant at 37°C, Cdc31p is no longer localized to the SPB (*H*). Cdc31p is relocalized to the SPB in *karl*Δ*17* at 37°C when the *CDC31-16* suppressor is present (*K*). Cdc31p also localizes to the SPB when *CDC31-16* is present in a complete *karl* deletion strain (*N*) as well as in a *KAR1*$^+$ background (*Q*). *A*, *D*, *G*, *J*, *M*, and *P* define the SPBs by anti-90-kD staining of the edge of the nucleus as shown by DAPI staining in *C*, *F*, *I*, *L*, *O*, and *R*. Cdc31p 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 Cdc31p but not by BSA. In addition, the interaction is more specific for Cdc31p than other calmodulin homologues because bovine calmodulin did not compete away binding at the concentrations tested. No major bacterial proteins were seen to bind Cdc31p, but there are additional Cdc31p-binding proteins in yeast that may be potential Cdc31p targets. Although the interaction between Cdc31p and Karlp did not appear to absolutely require calcium, we cannot exclude the possibility that labeling of Cdc31p 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 Cdc31p with Karlp requires the previously identified SPB domain of Karlp. Although Cdc31p 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 *KAR1* (Vallen et al., 1992*a*), as well as being both necessary and sufficient for the localization of Karlp-β-galactosidase hybrids to the SPB (Vallen et al., 1992*b*). 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 Cdc31p 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 Cdc31p 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-Cdc31p complex in vivo by other protein interactions.

Although wild-type Cdc31p 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 Cdc31p to be concentrated at the SPB. Under these conditions, the detection of Cdc31p 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 Cdc31p (Vallen et al., 1992*b*). It seems likely that the additional Cdc31p 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 Cdc31p at the SPB more accessible to the antibody. Nevertheless, these data show that an interaction between Karlp and Cdc31p occurs in vivo via the SPB domain of Karlp. In addition, we detected Cdc31p 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 Cdc31p is observed. It is likely that these fixation conditions make Cdc31p in the SPB more accessible to the antibody. While this manuscript was in preparation, Cdc31p localization to the SPB on isolated nuclei was reported using indirect immunofluorescence and immunoelectron microscopy (Spang et al., 1993). Their data suggested that Cdc31p localizes to the half-bridge, consistent with data showing a morphological change or loss of the half-bridge in *cdc31* and *kar1* mutants (Byers, 1981; Rose and Fink, 1987). Spang et al. (1993) suggested that Cdc31p and Karlp could not physically interact because of subtle differences in ultrastructural localization between Cdc31p and the protein aggregate including a Karlp-β-galactosidase hybrid protein. However, we have shown that Cdc31p 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 Cdc31p was no longer localized to the SPB in a *kar1* mutant that had a portion of the SPB domain deleted. This is additional in vivo evidence for an interaction between Karlp and Cdc31p, and it provides a suggestion for the normal in vivo function of Karlp.

Models for Karlp and Cdc31p Interaction

The SPB domain of Karlp was previously shown to be essential for SPB duplication. Because this domain interacts with Cdc31p 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 Cdc31p. We propose that the temperature sensitivity of *kar1Δ17* is caused by a decreased interaction with Cdc31p at the nonpermissive temperature. This idea is supported by immunofluorescence experiments that showed that Cdc31p was mislocalized in the *kar1Δ17* mutant. When a dominant *CDC31* suppressor allele was present, Cdc31p was relocalized to the SPB in *kar1Δ17*. Cdc31-16p was also localized to the SPB in a strain containing a complete deletion of *KAR1*. Therefore, it seems likely that Cdc31p needs to be localized to the SPB to carry out its essential function in SPB duplication. We propose that Karlp localizes Cdc31p to the SPB in wild-type cells, and the decreased interaction with Cdc31p in *kar1Δ17* caused Cdc31p to be mislocalized. The mechanism of *CDC31* suppression appears to involve the relocalization of Cdc31p to the SPB in the *kar1* mutants. Given that the *CDC31-16, kar1Δ2* strain grows normally, it would seem that *KAR1*'s only role in mitosis is to localize Cdc31p.

To explain the relocalization of the Cdc31p 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 *KAR1* 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 *KAR1* may be toxic (Vallen et al., 1994) because excess Cdc31p 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 *KAR1* might still require *KAR1* function, as well as increased interaction with another SPB component to localize Cdc31p to the SPB. This idea is supported by the observation that increased levels of wild-type Cdc31p suppress *kar1Δ17* (Vallen et al., 1994), possibly by stabilizing the interaction between the mutant Karlp and Cdc31p. However, it remains possible that the weaker suppressors act via an altered interaction with the mutant kar1Δ17 protein.

Our data and the results of Spang et al. (1993) have shown that Cdc31p localizes to both SPBs in wild-type cells. How-

ever, previous data showed that Karlp-β-galactosidase hybrids localize exclusively to the newly assembled SPB (Vallen et al., 1992*b*). Although this seems paradoxical, it is likely that the hybrid protein does not reflect the exact localization of wild-type Karlp because it forms an aggregate containing Cdc31p and other proteins. Possibly, the Karlp 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 Karlp hybrid aggregate may be lost from the old SPB because of cell cycle–regulated changes in interactions that reflect wild-type functions of Karlp. For example, Karlp 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 Karlp 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 Karlp is localized.

KAR1 and CDC31 Act in a Common SPB Duplication Pathway

We believe that *KAR1* 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 *kar1Δ17* suppressors, such as *DSK2-1* (Vallen et al., 1994). We have recently cloned the *dsk2*$^+$ 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 Ca^{2+}-binding proteins. *Proc. Natl. Acad. Sci. USA.* 83:5512–5516.

Byers, B., and L. 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. Stenderup, M. Kielland-Brandt, and J. Friis, editors. Munksgaard, Copenhagen. pp. 119–133.

Conde, J., and G. R. Fink. 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl. Acad. Sci. USA.* 73:3651–3655.

Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. *Cell.* 47: 423–431.

Davis, T. N., and J. Throner. 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.* 139:188–189.

Geiser, J. R., H. A. Sundberg, B. H. Chang, E. G. D. Muller, and T. N. Davis. 1993. The essential mitotic target of calmodulin is the 110-kilodalton 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 Ca^{2+}-binding protein. *J. Cell Biol.* 107:121–131.

Huang, B., A. Mengersen, and V. D. Lee. 1988b. Molecular cloning of cDNA for caltractin, a basal body-associated Ca^{2+}-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.* 106:1997–2010.

Jacobs, C. W., A. E. M. Adams, P. J. Szaniszio, and J. R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 107:1409–1426.

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. 1990. Mode of centriole duplication and distribution. *J. Cell Biol.* 110:1599–1605.

Kretsinger, 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, V. D., and B. Huang. 1993. Molecular cloning and centrosomal localization of human caltractin. *Proc. Natl. Acad. Sci. USA.* 90:11039–11043.

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.

Mirzayan, C., C. S. Copeland, and M. Snyder. 1992. The *NUF1* 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. *KAR1*, a gene required for function of both intranuclear and extranuclear 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. L. Satterwhite. 1993. Unraveling the tangled web at the microtubule-organizing center. *Curr. Opin. Cell Biol.* 5:105–115.

Rout, M. P., and J. V. Kilmartin. 1990. Components of the yeast spindle and spindle pole body. *J. Cell Biol.* 111:1913–1927.

Salisbury, J. L., A. T. Baron, and M. Sanders. 1988. The centrin-based cytoskeleton of *Chlamydomonas reinhardtii*: distribution in interphase and mitotic cells. *J. Cell Biol.* 107:635–641.

Schild, D., H. N. Anathaswamy, and R. K. Mortimer. 1981. An endomitotic effect of a cell cycle mutant of *Saccharomyces cerevisiae*. *Genetics.* 97: 551–562.

Spang, A., I. Courtney, 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.* 123:405–416.

Studier, 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.

Taillon, B. E., S. A. Adler, J. P. Suhan, 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. 1992*a*. Separate domains of *KAR1* 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. 1992*b*. Asymmetric mitotic segregation of the yeast spindle pole body. *Cell.* 69:505–515.

Vallen, E. A., W. Ho, M. Winey, and M. D. Rose. 1994. Genetic interactions between *CDC31* and *KAR1*, two genes required for duplication of the microtubule organizing center in *Saccharomyces cerevisiae*. *Genetics.* In press.

Winey, M., L. Goetsch, P. Baum, 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. *NDC1*: 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.