

The Cdc31p-binding Protein Kar1p Is a Component of the Half Bridge of the Yeast Spindle Pole Body

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Abstract. *KAR1* has been identified as an essential gene which is involved in karyogamy of mating yeast cells and in spindle pole body duplication of mitotic cells (Rose, M. D., and G. R. Fink. 1987. *Cell*. 48:1047-1060). We investigated the cell cycle-dependent localization of the Kar1 protein (Kar1p) and its interaction with other SPB components. Kar1p is associated with the spindle pole body during the entire cell cycle of yeast. Immunoelectron microscopic studies with anti-Kar1p antibodies or with the monoclonal antibody 12CA5 using an epitope-tagged, functional Kar1p revealed that Kar1p is associated with the half bridge or the bridge of the spindle pole body. Cdc31p, a Ca²⁺-binding protein, was previously identified as the first component of the half bridge of the spindle pole body (Spang, A., I. Courtney, U.

Fackler, M. Matzner, and E. Schiebel. 1993. *J. Cell Biol.* 123:405-416). Using an in vitro assay we demonstrate that Cdc31p specifically interacts with a short sequence within the carboxy terminal half of Kar1p. The potential Cdc31p-binding sequence of Kar1p contains three acidic amino acids which are not found in calmodulin-binding peptides, explaining the different substrate specificities of Cdc31p and calmodulin. Cdc31p was also able to bind to the carboxy terminus of Nuflp/Spcl10p, another component of the SPB (Kilmartin, J. V., S. L. Dyos, D. Kershaw, and J. T. Finch. 1993. *J. Cell Biol.* 123:1175-1184). The association of Kar1p with the spindle pole body was independent of Cdc31p. Cdc31p, on the other hand, was not associated with SPBs of *kar1* cells.

THE direction, number and polarity of microtubules of an eukaryotic cell is organized by microtubule organizing centers (MTOC)¹. The most prominent MTOC is the centrosome of higher eukaryotes. The spindle pole body (SPB) is the homologue of the centrosome in the yeast *Saccharomyces cerevisiae*. The SPB is a cylindrical multilaminated organelle which is embedded in the nuclear envelope (Byers and Goetsch, 1975). A number of substructures of the SPB are detectable by electron microscopy (see Fig. 9 A). The central plaque is in contact with the nuclear envelope. Inner and outer plaques function as nucleation sites for the nuclear and cytoplasmic microtubules, respectively (Byers and Goetsch, 1975; Byers, 1981a,b). The central plaque is disrupted by the less electron dense central line (Spang et al., 1993). An additional substructure of the SPB, the intermediate line, is located between the outer and central plaque (Rout and Kilmartin, 1990). The half bridge appears in the electron microscope as a darkly stained strip

along the cytoplasmic margin of the nuclear envelope on one side of the SPB (Byers and Goetsch, 1975; Byers, 1981a,b). The half bridge is not just a specialized section of the nuclear envelope, because it remains associated with enriched SPBs after the nuclear envelope is dissolved with detergent (Spang et al., 1993).

The SPB duplicates just once during the cell cycle of *Saccharomyces cerevisiae*. In early G₁, a structure called the satellite forms on the cytoplasmic face of the half bridge. After START of the cell cycle two SPBs can be observed side by side in the nuclear envelope. The duplicated SPBs are connected via the two half bridges, now called the bridge. At a later stage, the bridge is severed and the two separated SPBs form the poles of the mitotic spindle.

Several components of the SPB have been identified. Spindle or SPB components with molecular mass of 110, 90, and 80 kD were identified by raising monoclonal antibodies against an enriched fraction of SPBs (Rout and Kilmartin, 1990). The gene coding for the 110-kD SPB component was cloned independently by three different approaches (Mirzayan et al., 1992; Geiser et al., 1993; Kilmartin et al., 1993). The 110-kD SPB component (also called Nuflp and Spcl10p) has a predicted coiled-coil structure and regulates the spacing between the central and inner plaque of the SPB (Kilmartin et al., 1993). The COOH terminus of Nuflp/Spcl10p interacts in a Ca²⁺-dependent manner with calmodulin (CaM), another component of the SPB (Sun et

This paper is dedicated to Dr. Iain Courtney who died in July 1994.

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1. *Abbreviations used in this paper:* CDC, cell division cycle; DAPI, 4,6-diamidino-2-phenylindole; EGTA, bis-(aminoethyl)-glycoether-*N,N,N'*-tetraacetic acid; 5-FOA, 5-fluoroorotic acid; MTOC, microtubule organizing center; PCR, polymerase chain reaction; SPB, spindle pole body.

al., 1992; Geiser et al., 1993). The 90-kD component is associated with the outer and inner plaque of the SPB (Rout and Kilmartin, 1990). The 80-kD protein is bound to nuclear microtubules, particularly those close to the SPB (Rout and Kilmartin, 1990). Cdc31p—an essential Ca²⁺-binding protein—was identified as the first component of a SPB substructure, the half bridge (Baum et al., 1986; Spang et al., 1993). *KAR1* was originally identified by its role in karyogamy (Conde and Fink, 1976). Subsequent studies revealed its additional function in SPB duplication (Rose and Fink, 1987). These functions are mediated by two separate domains of Karlp. The NH₂-terminal section of Karlp seems to function in karyogamy, while the COOH terminus of Karlp is required for SPB duplication (Vallen et al., 1992a,b). The localization of wild-type Karlp was never determined. However, because overexpressed Karlp-β-galactosidase hybrid proteins localized to the SPB, it has been proposed that Karlp is a component of the SPB. One hybrid protein lacking the essential COOH terminus was localized by immunoelectron microscopy to the outer plaque of the SPB (Vallen et al., 1992b). In addition, this Karlp-β-galactosidase hybrid protein was associated with the SPB in a cell cycle-dependent manner. After SPB duplication, the hybrid protein was associated with only one of the two SPBs, usually the one that entered the bud (Vallen et al., 1992b). Recently, it was suggested that the localization of the Karlp-β-galactosidase proteins may not be identical at all stages with the localization of wild-type Karlp (Biggins and Rose, 1994).

In this study we investigated the subcellular localization of wild-type Karlp and the interaction of Cdc31p with Karlp.

Karlp is located to the SPB during the entire cell cycle of *Saccharomyces cerevisiae*. In addition, Karlp is associated with isolated SPBs and is a component of the half bridge or bridge of the SPB. Therefore, Karlp and Cdc31p localize to the same substructure of the SPB. The association of Karlp with the SPB was independent of Cdc31p. On the other hand, no Cdc31p was detected at the SPB in cells lacking Karlp. Both SPB components interact in vitro. Analysis of the sequence of Karlp which is responsible for Cdc31p binding suggests that Cdc31p and CaM have different binding requirements.

Materials and Methods

Yeast Strains, Media, Bacterial and Yeast Transformation

Table I summarizes the yeast strains that were used in this study. The standard medium for growth of yeast was yeast extract, peptone, dextrose growth medium (YPD). Synthetic minimal (SM) medium and synthetic complete (SC) medium were prepared as described by Guthrie and Fink (1991). *E. coli* strains were transformed by electroporation (Dower et al., 1988). Yeast cells were transformed using the lithium acetate method (Schiestl and Gietz, 1989).

DNA Techniques

DNA fragments were isolated from agarose gels with the Genclean II kit from Bio 101 (La Jolla, CA). PCR was performed with Vent polymerase supplied by New England Biolabs (Schwalbach, Germany). The nucleotide sequence of all PCR constructs was determined by the chain-termination method of Sanger et al. (1977). All other DNA manipulations were performed as described by Sambrook et al. (1989).

Table I. Yeast Strains

Strain	Genotype	Source or reference
S288C	<i>MATα mal gal2</i>	R. Mortimer (Univ. of California, Berkeley)
YPH499	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	Sikorski and Hieter (1989)
YPH501	<i>MATα/a ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1</i>	Sikorski and Hieter (1989)
UFM2	<i>MATα/a ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 Δkar1::HIS3/KAR1</i>	This study
ESM29	<i>MATα/a ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 Δkar1::HIS3/KAR1 {pSM30}*</i>	This study
ESM32	<i>MATα/a ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 Δkar1::HIS3/KAR1 {pBM12}‡</i>	This study
ESM33	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δkar1::HIS3 {pSM30}*</i>	This study
ESM34	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δkar1::HIS3 {pSM25}§</i>	This study
ESM36	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 kar1::HIS3 {pBM12}‡</i>	This study
ESM56	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 Δcdc31::HIS3 leu2-MET3-CDC31-LEU2</i>	Albermann, K., U. Fackler, A. Spang, K. Grein, and E. Schiebel (manuscript in preparation)
ESM115	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δkar1::HIS3 {pSM46} </i>	This study
Y49	<i>MATα cdc15-1 ura3 leu2 ade2 his3 trp1-1</i>	K. Nasmyth (I.M.P., Vienna)
Y255	<i>MATα leu2-3 leu2-112 cln1::HIS3 cln2 cln3::GAL-CLN3 cdc28-4 {p215}¶</i>	T. Schuster (Univ. of Würzburg)
BJ5626	<i>MATα/α ura3-52/ura3-52 trp1/TRP1 leu2Δ1/LEU2 his3Δ200/his3Δ200 pep4::HIS3/pep4::HIS3 prb1Δ1.6R/prb1Δ1.6R can1/can1</i>	E. Jones (Carnegie Mellon Univ., Pittsburgh)
MS2083	<i>MATα trp1-Δ1 leu2-3 leu2-112 ura3-52 kar1-Δ17</i>	Vallen et al. (1994)

* pSM30 is a pRS316 (Sikorski and Hieter, 1989) derivative that contains a restriction fragment of *KAR1*.

‡ pBM12 is a pRS316 derivative that contains *KAR1-HA*.

§ pSM25 is a pRS316 derivative containing *GST-KAR1*.

|| pSM46 is a pRS414 (Sikorski and Hieter, 1989) derivative containing *KAR1* under the control of the *MET3* promoter.

¶ p215 is a LEU2-based plasmid bearing *GAL-CLN1*.

Construction of *GST-KARI* and *GST-NUF1/SPC110* Gene Fusions, Construction of an Epitope-Tagged *KARI*, Construction of a *CDMI* Expression Plasmid

GST-KARI: The *KARI* gene of plasmid pMR76 (Rose and Fink, 1987) was amplified by PCR with the primers KARI-Bam (5'-CCGTGGGATCCATG-TAACTTCTCCAAA-3') and KARI-IV 5'-CGTAATCTGGAACGTCATATG-3'). The PCR product was restricted with BamHI and EcoRI and subcloned into the multiple cloning site of plasmid pGEX-3X (Pharmacia Biotech Inc., Piscataway, NJ) resulting in a gene fusion of glutathione-S-transferase (*GST*) and *KARI* (*GST-KARI*). The plasmid was named pSM9.

GST-ΔKARI: Plasmid pSM9 was restricted with BamHI and HindII. The 1,158-bp fragment was isolated and cloned into the BamHI, SmaI restriction sites of vector pGEX-3X. The recombinant plasmid generated was named pSM95. *GST-ΔKarl*p contained the NH₂ terminus (amino acids 1 to 386) of *Karl*p fused to the COOH terminus of *GST*.

GST-ΔΔKARI: pSM9 was restricted with BamHI and XmnI. The 702-bp fragment was cloned into pGEX-3X restricted with BamHI and SmaI. The plasmid generated was named pSM57. *GST-ΔΔKarl*p contains the NH₂ terminus (amino acids 1 to 237) of *Karl*p fused to the COOH terminus of *GST*.

GST-ΔKARI-Δ: The 456-bp XmnI-HindII fragment of *KARI* was cloned into the SmaI site of vector pGEX-5X (Pharmacia Biotech Inc.). The resulting plasmid was named pSM137. The *GST-ΔKarl*p-Δ fusion protein contains the amino acids 237 to 386 of *Karl*p fused to the COOH terminus of *GST*.

GST-ΔKARI-2Δ: Plasmid pSM137 was restricted with the enzymes NsiI and Sall. The Sall restriction site is located in the multiple cloning site of pGEX-5X and the NsiI restriction site in the coding region of *KARI*. Blunt ends were generated with T4 DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The vector was religated to give plasmid pSM148. *GST-ΔKarl*p-2Δ contains amino acids 237 to 350 fused to the COOH terminus of *GST*.

GST-ΔKARI-3Δ: Plasmid pSM137 was restricted with the restriction enzymes BsmI and Sall. BsmI is located in the open reading frame of *KARI*. The 3' and 5' ends were converted to blunt ends with T4 DNA polymerase. The vector was then religated to give plasmid pSM147. *GST-ΔKarl*p-3Δ contains amino acids 237 to 272 fused with the COOH terminus of *GST*.

GST-ΔKARI-4Δ: Plasmid pSM137 was restricted with the restriction enzyme XhoI. The linearized fragment was partially restricted with the enzyme RcaI. The ends were blunted with T4 polymerase and then religated. The construction was confirmed by sequencing. *GST-ΔKarl*p-4Δ contains amino acids 237 to 255 fused with the COOH terminus of *GST*.

KARI-HA: A NotI restriction site was constructed by PCR at the 3' end of the *KARI* gene using primers KARI-VIII (5'-GAATGCGGCCGCTAACCTATAATACATA-3') and KARI-IX (5'-GAACTGCGGCCGCTAAACCATATAAAGGTTAG-3'). The 114-bp NotI fragment of plasmid pGTEP-I (was kindly provided by Dr. Futcher, Cold Spring Harbor Lab., Cold Spring Harbor, NY) carrying three repeats which code for the hemagglutinin epitope (YPYDVPDYA) was inserted into the NotI restriction site at the 3' end of *KARI*. *KARI-HA* was cloned into pRS316 (Sikorski and Hieter, 1989) to give plasmid pBM12.

GST-N-NUF1: The 5' end of *NUF1/SPC110* (Mirzayan et al., 1992) was cloned by PCR using primers NUF1-1 (5'-CGTGGGATCCACGAAGCGTCACATCTC-3') and NUF1-2 (5'-TTTCTGAATCTTTGATTTCATGC-3') and chromosomal DNA from yeast strain S288C as a template. The DNA fragment was restricted with BamHI and EcoRI and cloned the BamHI-EcoRI sites of vector pGEX-3X. The plasmid was named pSM72. *GST-N-Nufl*p contains amino acids 2 to 175 of *Nufl*p/Spcl10p fused to the COOH terminus of *GST*.

GST-C-NUF1: The 3' end of *NUF1* was cloned by PCR using primers NUF1-3 (5'-GCTGGGATCCAGAGGCGTAAAGACAATG-3') and NUF1-4 (5'-CGTGGGATCCGAAGTTCAAAGAAGACTCC-3'). Chromosomal DNA from strain S288C was used as a template. The PCR product was restricted with BamHI and cloned into the BamHI site of vector pGEX-3X to give plasmid pSM74. *GST-C-Nufl*p contains amino acids 793-945 fused to the COOH terminus of *GST*. This region of *Nufl*p/Spcl10p carries the CaM binding site (amino acids 900 to 927, Geiser et al., 1993).

CDMI was amplified by PCR with the primers CaM-1 (5'-CGTGTACTGTGATATGA-3') and CaM-2 (5'-GTTATCCGCCTTGGTAA-3') using chromosomal DNA from strain S288C as a template. The PCR product was cloned into pUC18 with the Sure clone kit (Pharmacia, Freiburg, Germany). The resulting plasmid was named pSM61. The coding region of *CDMI* was then amplified by PCR with primers CaM-3 (5'-CCACGCATATGTCTTCCAATCTTAC-3') and universe (5'-GTAAACGACGGCCAGT-3'). Primer universe is complementary to a sequence in the multiple

cloning site of pUC18. The sense primer (CaM-3) was designed such that the translation initiation codon (ATG) of *CDMI* formed part of an NdeI restriction site. The PCR product was subcloned into the NdeI and PstI restriction sites of the expression vector pT7-7 (Tabor and Richardson, 1985) which generated a recombinant plasmid, named pSM71. Plasmid pSM71 expressed CaM under control of the T7 promoter.

Strain Constructions

UFM2 (KARI/Δkarl::HIS3). *KARI* was disrupted by the one-step gene replacement of Rothstein (1983). A 2,630-bp HindIII-EcoRI fragment of pMR76 (Rose and Fink, 1987) carrying the *KARI* gene was cloned into the HindIII-EcoRI sites of vector pBluescript SK (Stratagene, La Jolla, CA). The generated recombinant plasmid was named pSM11. A 882-bp NsiI fragment located in the coding region of *KARI* was substituted with the *HIS3* gene on a 1,157-bp PstI fragment. The resulting plasmid pUF4 was restricted with SacI and Sall; the fragments were transformed into the diploid yeast strain YPH501 (Sikorski and Hieter, 1989), selection being made on SC Media plates lacking histidine. The construction of the *UFM2* strain was confirmed by Southern analysis. Chromosomal DNA from His⁺ transformants (*UFM2*) and the parental strain (YPH501) was isolated and digested with restriction endonuclease HindIII and EcoRI. The fragments of DNA were separated on an agarose gel and transferred to a Hybond-N membrane (Amersham Buchler, Braunschweig, Germany) by vacuum blotting. The BglII-SacII fragment of pSM11 (which carries the entire *KARI* coding region) was labeled with digoxigenin and used as a probe in Southern analysis. Preparation of labeled probe, hybridization, and detection were performed with a kit from Boehringer Mannheim. One fragment which was 2,630-bp in length was detected with DNA from YPH501 using the *KARI* probe. With *UFM2*, two additional fragments were detected with 1,560- and 1,150-bp indicating that one chromosomal copy of *KARI* was substituted by the *HIS3* gene (data not shown).

ESM29 and ESM32. *UFM2* was transformed with plasmids pSM30 (carries the 2,630-bp HindIII-EcoRI *KARI*-fragment of pMR76 in pRS316) or pBM12 (*KARI-HA* in pRS316) selection being made on SC Medium plates lacking uracil. The transformants were named *ESM29* and *ESM32*, respectively.

ESM33 and ESM36. *ESM29* and *ESM32* were sporulated, and the spores from 20 tetrads were analyzed. Cells derived from spores of *ESM29* and *ESM32* were His⁺, Ura⁺, indicating that *KARI* on pSM30 and *KARI-HA* on pBM12 were functional. His⁺, Ura⁻ colonies were never obtained. Haploid colonies which were His⁺, Ura⁺ from the sporulation of *ESM29* and *ESM32* were named *ESM33 (Δkarl::HIS3 [pSM30])* and *ESM36 (Δkarl::HIS3 [pBM12])*, respectively. *ESM29* and *ESM32* were unable to grow on 5-fluoroorotic acid (5-FOA) plates suggesting that plasmids pSM30 and pBM12 were essential for the viability in these strains.

ESM115. The *KARI* gene was amplified by PCR with primers KARI-Nde (5'-ACGGACATATGATGTAATCTTCTCCAA-3') and KARI-IV (5'-CGTAACTCTGGAACGTCATATG-3'). Primer KARI-Nde is complementary to the 5' end of the *KARI* coding region. The 1,640-bp PCR fragment was treated with the Sure clone kit from Pharmacia (Freiburg, Germany) and ligated into the EcoRV site of plasmid pHAM8 (Mountain et al., 1991). Plasmid pHAM8 carries the *MET3* promoter in pUC19. The recombinant plasmid was named pSM45. A Sall-EcoRI DNA fragment carrying the *MET3-KARI* gene fusion was then cloned into the yeast *E. coli* shuttle vector pRS414 (Sikorski and Hieter, 1989). The resulting plasmid was named pSM46. Plasmid pSM46 was transformed into yeast strain *ESM33* selection being made on SC Medium plates lacking uracil and tryptophan. Transformants were streaked out on SM Medium plates which were supplemented with adenine, leucine, lysine, uracil, and 5-FOA. The *MET3* promoter is active in this growth medium which does not contain methionine. 5-FOA selects for cells which lost the *URA3*-based plasmid pSM30 (carries *KARI* under control of its own promoter). 5-FOA resistant colonies were Ura⁻ and unable to grow on plates containing methionine indicating that the essential *KARI* gene (Rose and Fink, 1987) is under control of the *MET3* promoter. This strain was named *ESM115 (Δkarl::HIS3 [pSM46])*.

ESM34. The *GST-KARI* gene fusion of plasmid pSM9 was amplified by PCR using primers KARI-B (5'-ACTCGCGCGGATCCACTTCGATC-ACTTT-3') and KARI-C (5'-GATACTGCGGCCGCTCCCTATACTGA-3'). KARI-B binds to the 3' non-coding region of *KARI*. KARI-C is complementary to the 5' end of the *GST* coding region. The PCR product was restricted with NotI and cloned into the NotI restriction site of plasmid pSM13 to give plasmid pSM25. Plasmid pSM13 was constructed as follows. The 5' non-coding region of *KARI* was amplified with primers KARI-I (5'-ACGAAG-GATCCTTTGAATATCAATGAAAAT-3') and KARI-II (5'-GATACTGCG-GCCGCTCATTCATGAACCAAAA-3'). The PCR product was restricted

with BamHI and NotI and then cloned into the BamHI and NotI sites of the vector pRS316 (Sikorski and Hieter, 1989). Yeast strain UFM2 (*Karl::His3*) was transformed with plasmid pSM25 selection being made on SC plates lacking uracil. Transformants were sporulated and tetrads dissected. Colonies derived from spores of UFM2 (pSM25) which were Ura^r, His⁺ (*Δkarl::His3* [pSM25]) were obtained with high frequency (strain ESM34).

Nuclei Isolation, SPB Preparation, Cell Fractionation, Cell Extracts

Nuclei and SPBs from yeast strain BJ5626 or ESM36 were isolated as described by Rout and Kilmartin (1990). Cells of the diploid yeast strain BJ5626 were fractionated into a cell lysate, a soluble and an insoluble fraction as described by Spang et al. (1993). Protein extracts were prepared as described by Ausubel et al. (1994).

Isolation of the GST-Karlp and GST-Nuflp Fusion Proteins

Expression of GST-Karlp in *E. coli* BL21 (DE3, pSM9) (Studier and Moffat, 1986) was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG). DE3 of *E. coli* strain BL21 (DE3) indicates that the strain carries a lysogenic lambda phage with the T7 RNA polymerase structural gene (Studier and Moffat, 1986). The *E. coli* cells were lysed after 3 h of induction by ultrasonication as described by Spang et al. (1993). The GST-Karlp fusion protein formed inclusion bodies which were enriched by differential centrifugation. Unlysed cells were removed by low speed centrifugation (10 min, 1,000 g, 4°C). Inclusion bodies were then collected by centrifugation (15 min, 10,000 g, 4°C). The GST-Karlp fusion protein was further purified by preoperative SDS-PAGE (Laemmli, 1970). The separated proteins were transferred onto nitrocellulose and stained with Ponceau S (Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany).

Expression and purification of GST- Δ Karlp, GST- $\Delta\Delta$ Karlp, GST- Δ Karlp-2 Δ , GST- Δ Karlp-3 Δ , GST- Δ Karlp-4 Δ and GST-N-Nuflp in *E. coli* BL21 (DE3), containing the corresponding plasmids were as recommended by Pharmacia.

Preparation of Affinity-purified Anti-Karlp, Anti-N-Nuflp, Anti- β -Tubulin Antibodies

GST-Karlp containing nitrocellulose strips were dissolved with DMSO and injected into rabbits as described by Harlow and Lane (1988). Purified anti-Karlp antibodies were prepared by affinity chromatography as described by Harlow and Lane (1988). An *E. coli* extract containing GST was coupled to CNBr-Sepharose as recommended by Pharmacia. The IgG fraction containing the anti-Karlp antibodies was run over the GST *E. coli* protein column to remove anti-GST antibodies. The preabsorbed IgG fraction was then incubated with purified GST- Δ Karlp coupled to CNBr-Sepharose 4B. Anti-Karlp antibodies were eluted with 100 mM glycine, pH 2.5. The pH was immediately adjusted to pH 7.5 with 1 M Tris-HCl, pH 8.0. BSA (0.1 mg/ml) was added to the affinity-purified anti-Karlp antibodies which were dialyzed against phosphate-buffered saline.

Purified GST-N-Nuflp was injected into a goat (Harlow and Lane, 1988). Purified anti-N-Nuflp antibodies were prepared as described for anti-Karlp antibodies using purified GST-N-Nuflp coupled to CNBr-Sepharose.

Goat anti- β -tubulin peptide antibodies were prepared as follows: a peptide (CMSKNRSSLQSGPLN) corresponding to the COOH-terminal amino acids of yeast β -tubulin was coupled to BSA with m-maleimido-benzoyl-N-hydroxysuccinimide ester (MBS; Pierce, Rockford, IL). The coupled peptide was injected into a goat as recommended by Harlow and Lane (1988). For affinity-purification of the anti- β -tubulin antibodies, the β -tubulin peptide was coupled to epoxy-activated Sepharose 6B as recommended by Pharmacia. Anti- β -tubulin antibodies were eluted from the peptide resin with 100 mM glycine, pH 2.5. The pH of the eluted antibodies was adjusted immediately as described for the anti-Karlp antibodies. The anti- β -tubulin antibodies were specific for β -tubulin in immunoblots and immunofluorescence.

Immunoelectron Microscopy, Immunofluorescence of Nuclei and Yeast Cells

Immunoelectron microscopy of whole yeast cells and isolated SPBs was performed with affinity-purified anti-Karlp or 12CA5 antibodies (Hiss Diagnostics GmbH, Freiburg, Germany) as described by Preuss et al. (1991)

with the modifications of Spang et al. (1993). A 1:500 dilution of anti-Karlp and a 1:250 dilution of 12CA5 antibodies were used. The gold particles were visualized in a Jeol JEM 100B electron microscope (Jeol Ltd., Tokyo, Japan).

Immunofluorescence of yeast nuclei and yeast cells were performed as described by Spang et al. (1993) and Adams and Pringle (1984). Cells were fixed for 5 min with formaldehyde. A 1:1,000 dilution of affinity-purified anti-Karlp antibodies was used. A pool of various mouse monoclonal anti-90-kD antibodies was a generous gift from Dr. J. Kilmartin. Secondary antibodies were goat anti-rabbit IgG conjugated with Cy3 and goat anti-mouse IgG conjugated with FITC from Jackson ImmunoResearch Laboratories (West Grove, PA). No signal was obtained when nuclei or cells were incubated either with secondary antibodies only or with preimmune serum. Similarly, no signal was obtained in double-labeling experiments in which treatment with either of the primary antibodies was followed by incubation with the "mismatched" secondary antibody. Immunofluorescence microscopy was performed with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Immunoblotting, Protein Determination

Protein concentration of samples was determined using the method from Bradford (1976). Proteins were separated by SDS-PAGE as described by Laemmli (1976). Immunoblotting was performed as described by Spang et al. (1993). Detection was made by enhanced chemiluminescence (ECL) using a kit from Amersham Buchler GmbH (Braunschweig, Germany). The primary antibodies were rabbit anti-Cdc31p (Spang et al., 1993), rabbit anti-Karlp, goat anti-Nuflp/Spcl10p (anti-110-kD protein), goat anti- β -tubulin and mouse anti-90-kD antibodies. In the case of the mouse monoclonal anti-90-kD antibody, incubation with the primary antibody was followed by an incubation with rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories). Secondary antibodies were goat anti-rabbit IgG (Bio Rad, Hercules, CA) and rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories) conjugated with horseradish peroxidase.

Isolation of [³⁵S]Cdc31p and [³⁵S]CaM

E. coli strain BL21 (DE3, pLysS, pSM5) (Spang et al., 1993) or BL21 (DE3, pLysS, pSM71) were grown in TY medium (bacto trypton, yeast extract) supplemented with 100 μ g/ml ampicillin to mid-log phase. Plasmid pSM5 (Spang et al., 1993) and pSM71 contain *CDC31* and *CDM1*, respectively, under the control of the inducible phage T7 promoter (Tabor and Richardson, 1985). Plasmid pLysS carries the T7 lysozyme gene. T7 lysozyme inhibits residual amounts of the T7 RNA polymerase before induction with IPTG (Studier and Moffat, 1986). The *E. coli* cells were washed three times with M9 minimal medium and then incubated for 1 h at 37°C. IPTG (0.5 mM) was added to the cultures to induce the T7-RNA polymerase. Cells were incubated for 30 min at 37°C. Finally, rifampicin (200 μ g/ml) was added to inhibit the *E. coli* RNA polymerase. Cells were incubated for 30 min at 37°C. Cdc31p and CaM were selectively labeled by the addition of 10 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine (Tran-³⁵S-label; DuPont, Bad Homburg, Germany) for 5 min at 37°C.

Cells were harvested by centrifugation (3 min, 14,000 g, 4°C). The cell sediment was resuspended in 1/10 vol 50 mM Tris-HCl, pH 8, 0.5 mM EDTA, 1 mM PMSF, 100 μ g/ml lysozyme. The cells were lysed by three freeze-thawing cycles. Unlysed cells were removed by centrifugation (14,000 g, 4°C, 15 min). Cdc31p and CaM were then enriched by heating the cell extract for 5 min at 80°C. Denatured proteins were removed by centrifugation (14,000 g, 4°C, 15 min). 90% of Cdc31p and CaM was in the supernatant. The enriched Cdc31p and CaM were radiochemically pure. The chemical purity of Cdc31p and CaM was ~80% as estimated by SDS-PAGE.

Interaction of Cdc31p and CaM with Karlp and Nuflp/Spcl10p

E. coli extracts containing GST-Karlp, GST- Δ Karlp, GST- $\Delta\Delta$ Karlp, GST-N-Nuflp and GST-C-Nuflp were separated by SDS-PAGE. The extracts were transferred onto a nitrocellulose membrane by electroblotting using a semi-dry transfer cell from Bio Rad. Protein binding sites were blocked with 20 mM Hepes, pH 7.5, 200 mM NaCl, 5% BSA, 0.05% Tween for 2 h at 20°C. Membranes were then incubated either with buffer A (20 mM Hepes, pH 7.5, 200 mM NaCl, 0.05% Tween, 0.1% BSA, 0.1% gelatine, 1 mM CaCl₂) or buffer B (20 mM Hepes, pH 7.5, 200 mM NaCl, 0.05% Tween, 0.1% BSA, 0.1% gelatine, 10 mM EGTA) for 1 h at 20°C. Purified [³⁵S]Cdc31p (100,000 cpm/ μ g) or [³⁵S]CaM (100,000 cpm/ μ g) was in-

cubated with buffer A or B for 1 h at 4°C. The preincubated Cdc31p and CaM were incubated with the nitrocellulose membranes in buffer A or B for 2 h at 20°C. To remove unbound Cdc31p and CaM, the membranes were washed four times with buffer A or B, respectively, for 20 min at 20°C. The dried membranes were autoradiographed using a X-omat AR film from Kodak.

Dot Blot Overlay Assay

GST-ΔKarl_p, GST-ΔKarl_p-Δ, GST-ΔKarl_p-2Δ, GST-ΔKarl_p-3Δ, GST-ΔKarl_p-4Δ, and GST were expressed in *E. coli* BL21 (DE3) and purified as described. 50 pmol of the various GST fusion proteins were immobilized onto nitrocellulose membranes using a dot blot apparatus from Schleicher and Schuell (Dassel, Germany). The membrane was incubated with [³⁵S]Cdc31p in buffer A for 2 h at 20°C. The membrane was washed four times with buffer A for 20 min at 20°C. Sections of nitrocellulose membranes containing the fusion proteins and bound [³⁵S]Cdc31p were cut out. The radioactivity was determined by a liquid scintillation counter (LS1801; Beckman, Palo Alto, CA). One sample did not contain any protein. The radioactivity bound to this sample was considered as nonspecific binding. The other samples were corrected for the non-specific binding. All experiments were performed in triplicates.

Synchronization of Yeast Cells Using Strain Y255

Strain Y255 carries the *cdc28-4* allele and in addition *chl1::HISG* and *chl2Δ*. The *CLN1* and *CLN3* genes are under control of the *GALI* promoter (*GALI::CLN1* on plasmid p215(LEU2) and *GALI::CLN3* in the *CLN3* locus) (T. Schuster, unpublished observation). Y255 was grown in SC Medium containing 2% raffinose and 1% galactose to mid log phase. The cells were washed twice with water and resuspended in SC medium with 2% raffinose. After 4 h in SC raffinose medium >95% of the cells were arrested at START. The cells continued synchronously in the cell cycle after the addition of 2% galactose to the SC raffinose medium.

Results

Karl_p Is Associated with the SPB During the Entire Cell Cycle of Yeast *Saccharomyces cerevisiae*.

The yeast *KAR1* is an essential gene which is required for SPB duplication and nuclear fusion (Rose and Fink, 1987; Conde and Fink, 1976). Despite its importance, the cellular distribution of wild-type Karl_p has not been established. For this reason, we investigated the localization of wild-type Karl_p with affinity-purified anti-Karl_p antibodies using immunofluorescence and immunoelectron microscopy.

The specificity of the antibody-antigen interaction was critical for the success of these experiments. Affinity-purified anti-Karl_p antibodies were prepared as described in Materials and Methods. In a total yeast extract of strains BJ5626 and ESM33 expressing wild-type Karl_p, only one protein was recognized by the affinity-purified anti-Karl_p antibodies (Fig. 1, lanes 1 and 2). This protein had a molecular mass of 53 kD which is the predicted MW of Karl_p (Rose and Fink, 1987). The Karl_p-HA and GST-Karl_p fusion proteins of strains ESM36 (lane 3) and ESM34 (lane 5) should have molecular masses of 56 and 79 kD, respectively. In agreement with this prediction, proteins with these molecular masses were detected from extracts of ESM36 and ESM34. A small amount of Karl_p was detected in some preparations extracted from ESM36 which probably reflects proteolytic degradation of Karl_p-HA during sample preparation. Strain MS2083 carries the *kar1-Δ17* mutation (Vallen et al., 1992b; 1994). As expected for this deletion, the anti-Karl_p antibodies detected a 49-kD protein in extracts of MS2083 (lane 4). These results demonstrate that our anti-Karl_p antibodies are highly specific for Karl_p and suitable

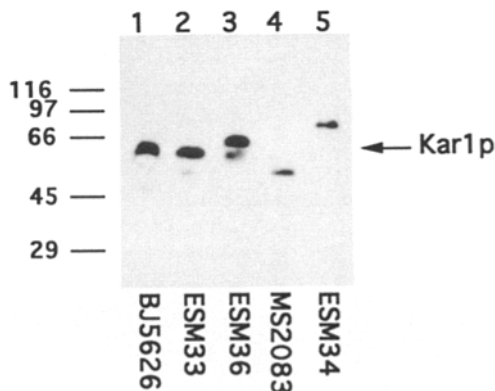


Figure 1. The affinity-purified anti-Karl_p antibodies are specific. Total yeast extracts were prepared as described in Materials and Methods from yeast strains BJ5626 (*KAR1*, lane 1), ESM33 (*KAR1*, lane 2), ESM36 (*KAR1-HA*, lane 3), MS2083 (*kar1-Δ17*, lane 4) and ESM34 (*GST-KAR1*, lane 5). Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane and incubated with affinity-purified anti-Karl_p antibodies. The immunoblot was developed with an ECL kit from Amersham.

for localization studies by indirect immunofluorescence and immunoelectron microscopy.

The localization of Karl_p in yeast strain BJ5626 was investigated by indirect immunofluorescence. Double-labeling experiments were performed with affinity-purified anti-Karl_p and anti-90-kD antibodies. The monoclonal anti-90-kD antibodies were used as markers for SPBs (Rout and Kilmartin, 1990). DNA was stained with DAPI. As expected for SPB components, the Karl_p and the 90-kD proteins were detected as one or two dots at the nuclear periphery (Fig. 2 A, compare anti-Karl_p and anti-90-kD with DAPI). No defined Karl_p staining was observed in other parts of the cell.

To confirm the nuclear localization of Karl_p, the immunofluorescence experiment was repeated with isolated nuclei (Fig. 2 B). In agreement with the immunofluorescence of yeast cells, the signal obtained with the anti-Karl_p and anti-90-kD antibodies appeared as one or two dots at the nuclear periphery (Fig. 2 B, compare anti-Karl_p and anti-90-kD with DAPI staining). In 98% of the cases ($n = 1000$), the Karl_p and 90-kD signals coincided, indicating an association of Karl_p with the SPB. In ~1% a double spot of anti-Karl_p or anti-90-kD gives a single signal with the other antibody. In the remaining 1% a SPB staining was only obtained with either the anti-90-kD or the anti-Karl_p antibodies.

The Karl_p-β-galactosidase hybrid proteins showed a typical cell cycle-dependent behavior (Vallen et al., 1992b). After SPB duplication, the hybrid proteins were found in association with only one of the two SPBs, usually that SPB which enters the bud (Vallen et al., 1992b). We investigated whether wild-type Karl_p is also associated with the SPB in a cell cycle-dependent manner. At first, the percentage of SPBs of monopolar, short and long spindles, which were detected by the anti-Karl_p and anti-90-kD antibodies, were determined (Table II). Most (>95%) of the SPBs were costained by the anti-Karl_p and anti-90-kD antibodies independent of the cell cycle stage of the yeast cells. In a few cells no colocalization was observed (Table II). Second, we synchronized yeast cultures and determined the distribution

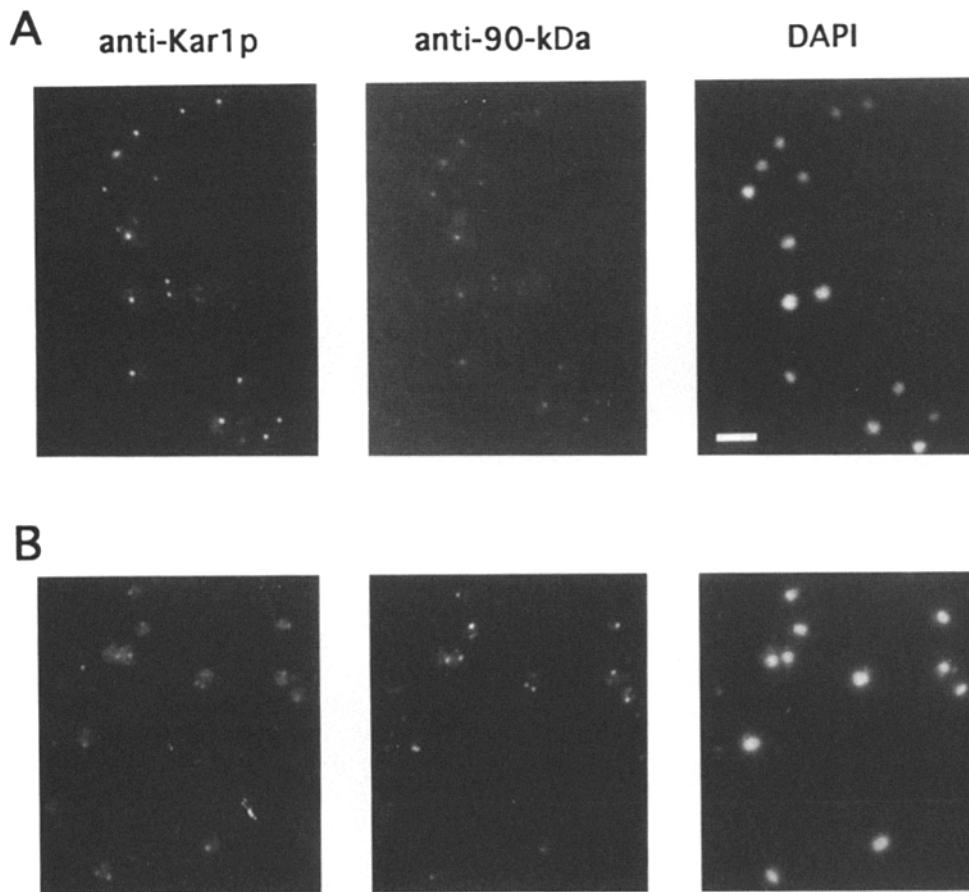






Figure 2. Kar1p is associated with the SPB. Immunofluorescence microscopy of whole yeast cells (*A*) and isolated nuclei (*B*). (*A*) Whole yeast cells from strain BJ5626 were fixed and then stained with affinity-purified rabbit anti-Kar1p and monoclonal mouse anti-90-kD antibodies. (*B*) Yeast nuclei from strain BJ5626 were incubated with affinity-purified anti-Kar1p antibodies and monoclonal 90-kD antibodies. Secondary antibodies used in *A* and *B* were described in Materials and Methods. DNA was stained with DAPI. Magnification in *A* is the same as in *B*. Bar, 5 μ m.

of Kar1p by indirect immunofluorescence. To exclude possible artifacts, yeast cells were synchronized by two different methods, using either a *cdc15* mutant or strain Y255. Since both approaches yielded the same result, only the data obtained with the *cdc15* cells are shown (Fig. 3). Cells of *cdc15* arrest uniformly at the non-permissive temperature in mitosis with duplicated SPB and a long mitotic spindle (Fig. 3, 0 min). Clearly, both SPBs were stained by the anti-Kar1p

antibodies at this stage of the cell cycle. Cells of *cdc15* were then shifted to permissive conditions, whereupon the cells continued in the cell cycle. After the release of the cell cycle block, samples were taken every 30 min and the localization of Kar1p was determined by indirect immunofluorescence. Kar1p was associated with the SPB in yeast cells with no buds (Fig. 3, G₁ phase of the cell cycle, 30 min after the temperature shift), small- (S-phase, 60 min), medium- (G₂, 90 min), and large-budded cells (mitosis, 120 min). These results strongly suggest that Kar1p is a constitutive component of the yeast SPB.

Table II. Distribution of Kar1p and the 90-kD SPB Component during the Cell Cycle

				
Number of cells investigated	74	73	61	110
1 dot per cell				
colocalization of Kar1p/90 kD	100%	47%	2%	1%
2 dots per cell				
colocalization of Kar1p/90 kD	—	49%	92%	99%
Partial colocalization of Kar1p/90 kD (1 dot with colocalization and 1 dot without colocalization)	—	3%	5%	0%
No colocalization of Kar1p/90 kD	0%	1%	1%	0%

An early logarithmic growing culture of yeast strain BJ5626 was prepared for immunofluorescence as described in Materials and Methods and stained with anti-Kar1p and anti-90-kD antibodies. The DNA was visualized with DAPI. SPB staining appeared as one or two dots dependent on the cell cycle stage of the yeast cell (also see Figs. 2 and 3).

Kar1p Is a Component of the Half Bridge of the SPB

To understand the function of SPB components and the structure of the SPB, it is important to determine the association of SPB components with substructures of this organelle. At least nine structures of the SPB are detectable by electron microscopy—outer, inner, and central plaque, intermediate and central line, cytoplasmic and nuclear side of the half bridge and bridge, satellite, and connecting fibers between the inner and central plaque. Despite its importance, the sub-localization of only three SPB components—Nuf1p/Spc110p, 90-kD component, and Cdc31p—have been determined by immunoelectron microscopy (Rout and Kilmartin, 1990; Kilmartin et al., 1993; Spang et al., 1993).

We first studied the localization of Kar1p by immunoelectron microscopy using isolated SPBs. Enriched fractions of SPBs contain about 500 \times more SPBs per volume in comparison to whole yeast cells, making it much easier to obtain

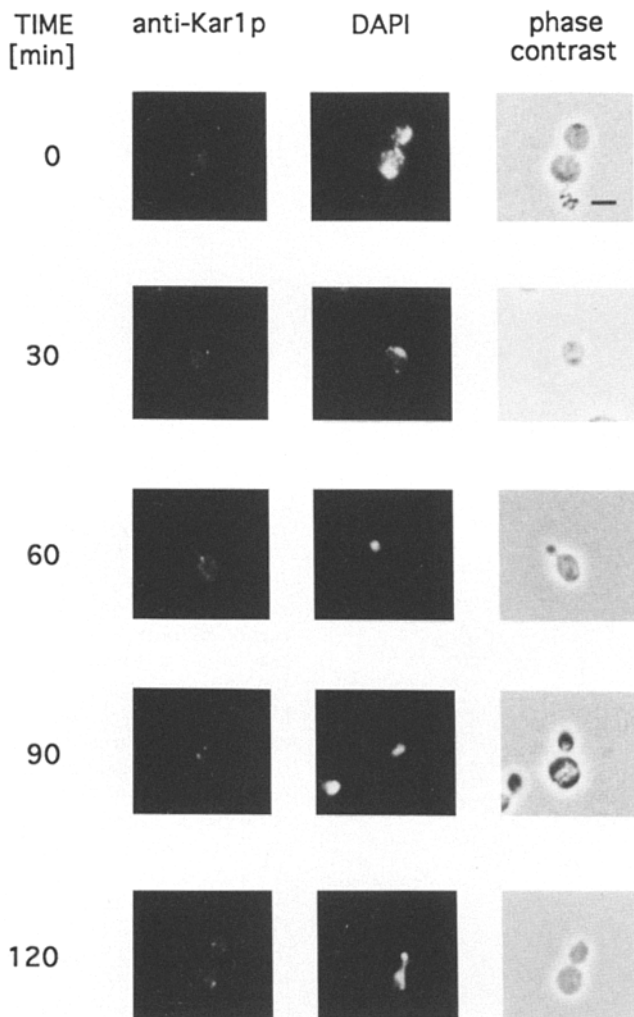


Figure 3. Karlp is associated with the SPB during the entire cell cycle. Strain Y49 (*cdc15-1*) was grown in YPD at 23°C to mid-log phase. The culture was then shifted to 37°C until >90% of the cells were arrested as large-budded cells. The arrested cells continued in the cell cycle after decreasing the incubation temperature to 23°C ($t = 0$). Samples were taken every 30 min and analyzed by immunofluorescence with anti-Karlp antibodies and phase contrast microscopy. DNA was stained with DAPI. Only one representative cell is shown. However, >85% of the cells had the indicated phenotype. Bar, 5 μ m.

a representative view of the localization of a SPB component. However, it was important to establish that the SPB component of interest does not disassemble from the SPB during the enrichment procedure. Therefore, we tested whether Karlp cofractionates with enriched preparations of SPBs. Equal amounts of total protein from a cell lysate, 100,000 *g* supernatant and sediment fractions, isolated nuclei, sucrose and Percoll gradient-enriched SPBs were separated by SDS-PAGE. The distribution of three SPB components, Cdc31p (Fig. 4 A), Nuf1p/Spc110p (Fig. 4 B), and 90-kD SPB component (Fig. 4 C) was determined as markers for the enrichment of SPBs. In addition, β -tubulin (Fig. 4 D) and two nucleolar proteins, Nop1p and Nop2p (Jansen et al., 1993) (Fig. 4 F), were also detected by immunoblotting. As we expected, the SPB components Nuf1p/Spc110p, 90-kD protein and Cdc31p were most strongly enriched in the frac-

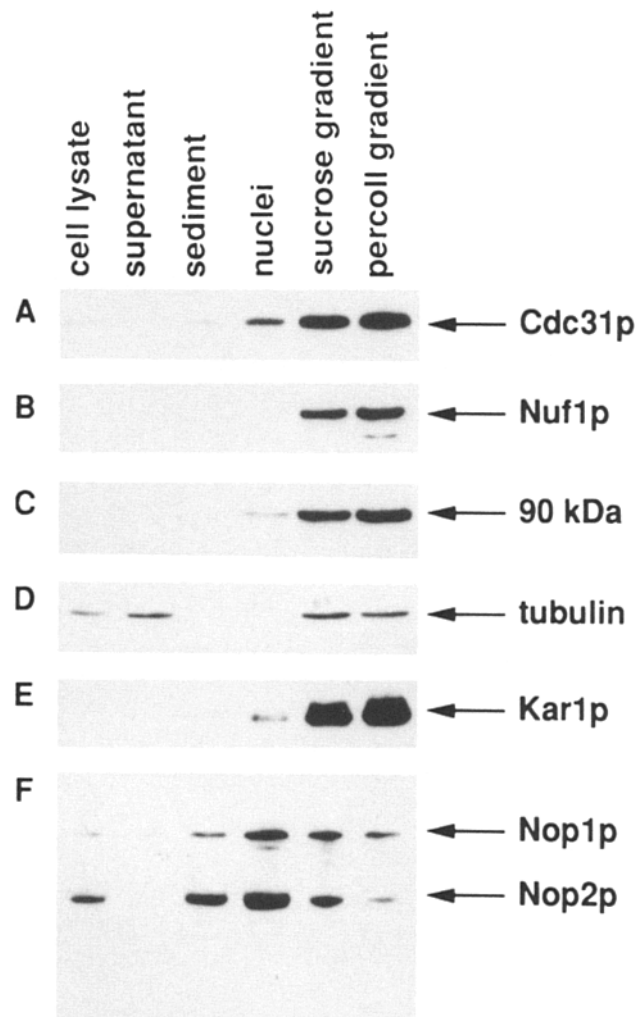


Figure 4. Karlp cofractionates with SPBs. A cell lysate from strain BJ5626 was fractionated into 100,000 *g* supernatant and sediment, isolated nuclei, and enriched SPBs (sucrose and Percoll gradient). Equal amounts (10 μ g) of protein per lane were analyzed by SDS-PAGE and subsequent immunoblotting using primary antibodies directed against: (A) Cdc31p, (B) Nuf1p/Spc110p, (C) 90-kD SPB component, (D) β -tubulin, (E) Kar1p, and (F) the nucleolar proteins Nop1p and Nop2p. The immunoblots were developed as described in Fig. 1.

tions containing most of the SPBs (sucrose and Percoll gradient purified SPBs). The amount of tubulin decreased at first with the enrichment of nuclei. This is in agreement with the observation that purified nuclei contain almost no microtubules, since most microtubules were lost during the isolation of the nuclei (Spang et al., 1993). Tubulin was strongly enriched with the SPBs from the sucrose gradient, as expected for a SPB-associated component, then partially separated from SPBs from the Percoll gradient. This reflects a decrease in length of the microtubules associated with the SPBs from the sucrose in comparison to the SPBs of the Percoll gradient (data not shown). Nop1p and Nop2p were most strongly enriched with isolated nuclei, as expected for proteins of the nucleolus. The concentration of these proteins subsequently decreased with increasing purity of the SPBs, indicating a specific enrichment of SPBs. Karlp was progres-

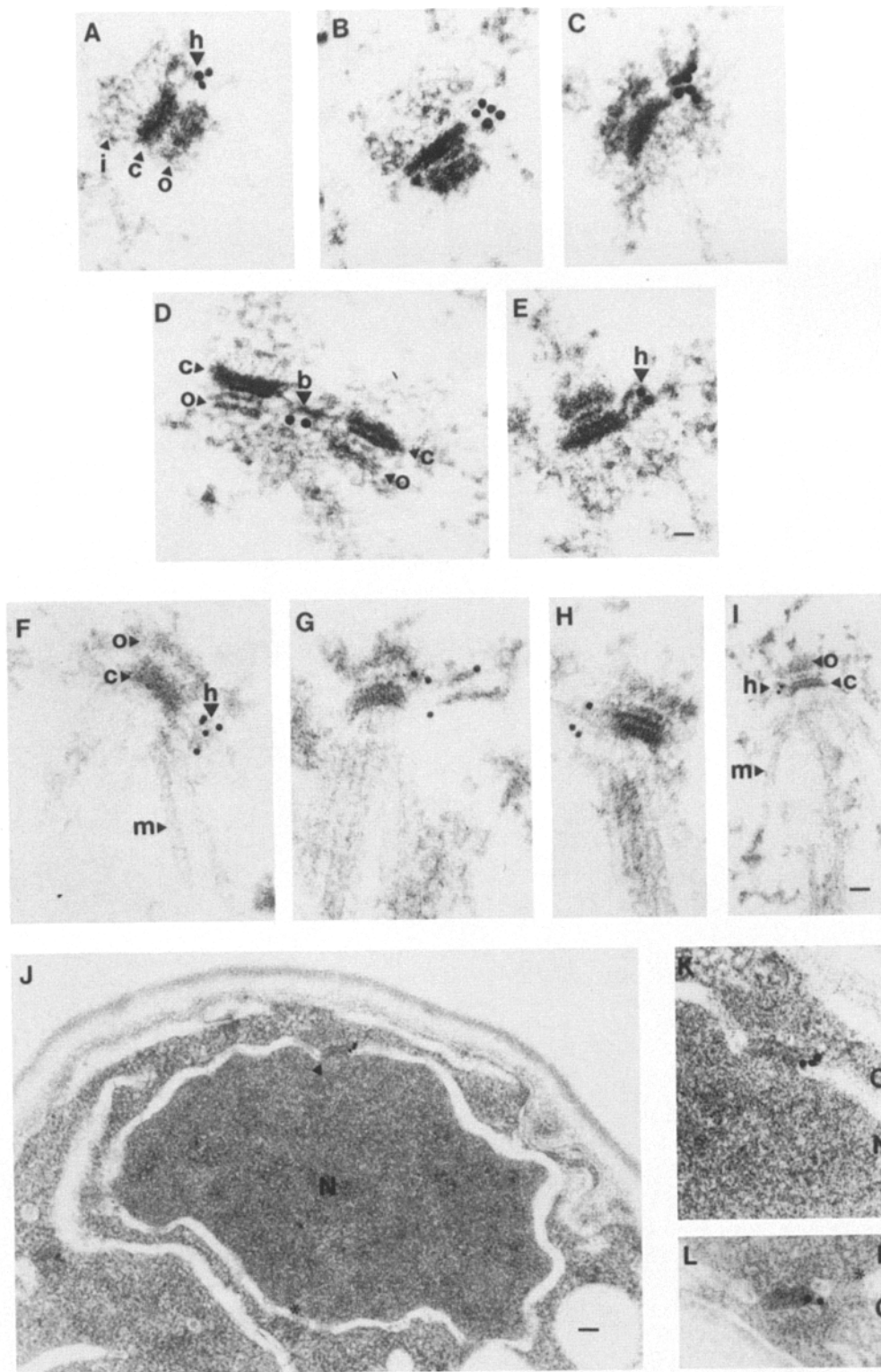


Figure 5. Karlp is a component of the half bridge of the SPB. Immunoelectron microscopy of isolated SPBs (A to I) and whole yeast cells (J to L). (A-E) SPBs were purified from strain BJ5626 and labeled with affinity purified anti-Karlp antibodies. Single SPBs are shown in (A-C, and E). (D) Mother and daughter SPBs connected by the bridge. (F-I) SPBs were isolated from strain ESM36 (*KAR1-HA*) and labeled with 12CA5 monoclonal antibodies. (J-L) Whole cells of ESM36 were labeled with 12CA5 antibodies. (J) Nucleus with SPB. (K) Enlargement of J. (L) A single SPB next to a nuclear pore. *b*, bridge; *c*, central plaque; *C*, cytoplasm; *h*, half bridge; *i*, inner plaque; *m*, microtubules; *N*, nucleus; *o*, outer plaque. The arrow in J indicates the position of the central plaque of the SPB in the nuclear envelope. The asterisk in J and L indicates the position of a nuclear pore. Bars: (E) 31 nm; (I) 62 nm; (J) 65 nm. (A-H and K, L) are the same magnification as E.

sively enriched with increasing purity of the SPBs and behaved very like the SPB components Cdc31p, Nuflp/Spcl10p and 90-kD protein. We conclude from this result that Karlp cofractionates with purified preparations of SPBs.

The localization of Karlp was investigated by immunoelectron microscopy with ultra-thin sections of enriched SPBs using affinity-purified anti-Karlp antibodies. The substructures of the SPB—outer, central, and inner plaque and half

bridge or bridge—were clearly detectable (Fig. 5, A-E). No cytoplasmic or nuclear microtubules were associated with the SPBs in this preparation. Two to six gold particles were associated with an electron dense structure—the half bridge—next to the central plaque (Fig. 5, A-C and E). After START of the cell cycle, the SPB duplicates and the two SPBs are still connected by the bridge. The bridge appeared in electron micrographs as an electron dense layer connect-

ing the central plaques of the mother and daughter SPB (Fig. 5 D). Gold particles were localized with the cytoplasmic side of the bridge (Fig. 5 D). Only ~10% of the isolated SPBs were labeled by the anti-Karlp antibodies. In these cases, the half bridge or bridge was stained. No staining of other structures was observed. One possible explanation for the poor efficiency of the SPB staining by the anti-Karlp antibodies is the fixation-sensitivity of Karlp epitopes. In agreement with this conclusion, we observed that the percentage of SPBs stained by immunofluorescence of yeast cells strongly decreased with increasing fixation time (data not shown).

To confirm the association of Karlp with the half bridge, we repeated the immunoelectron microscopy with an epitope-tagged Karlp-HA protein. A gene fusion consisting of *KARI* and three sequences coding for the hemagglutinin epitope (YPYDVPDYA) was constructed. The plasmid coding for *KARI-HA* (pBM12) was transformed into the yeast strain UFM2 (*KARI/Δkarl::HIS3*). The strain UFM2 (pBM12) was sporulated, and the viability of 20 tetrads was determined. Colonies from spores which were haploid, His⁺ and Ura⁺ were obtained, indicating that these strains carried the *Δkarl::HIS3* disruption and the *URA3*-based plasmid pBM12. This strain was designated ESM36. Since *KARI* is essential for viability (Rose and Fink, 1987) and the chromosomal *KARI* gene of ESM36 is disrupted, *KARI-HA* on plasmid pBM12 must provide *KARI* function. Immunoblotting of cell extracts of ESM36 with anti-Karlp (Fig. 1, lane 3) and 12CA5 (data not shown) antibodies revealed the Karlp-HA fusion protein with the expected molecular mass of ~58 kD. In a wild-type control (YPH499), however, Karlp (molecular mass of 53 kD) was detected with the anti-Karlp but not the 12CA5 antibodies. In addition, we obtained SPB staining by indirect immunofluorescence using ESM36 cells and 12CA5 antibodies. A wild-type strain did not show any signal with 12CA5 antibodies (data not shown). Taken together, these results indicate that the Karlp-HA fusion protein is expressed in ESM36 cells, provides Karlp function, and localizes with the SPB.

We enriched SPBs from ESM36 cells and determined the localization of Karlp-HA by immunoelectron microscopy using 12CA5 antibodies (Fig. 5, F-I). In this experiment, the embedded and sectioned SPBs still contained microtubules. More than 50% of the SPBs were labeled by the 12CA5 antibodies. The gold particles were again associated with the half bridge, confirming our results with the anti-Karlp antibodies. SPBs from a wild-type strain were not stained with the 12CA5 antibodies (data not shown).

To confirm the localization of Karlp-HA in whole yeast cells, sections of cells of ESM36 were labeled with 12CA5 antibodies (Fig. 5, J-L). The SPBs were sectioned so that the central plaque of the SPB is clearly visible in a discontinuity of the nuclear envelope. Also visible in the micrograph is the half bridge as an electron-dense extension of the central plaque of the nuclear envelope. The outer and inner plaques were not detected by the procedure used for immunoelectron microscopy. Most of the yeast cells contained one or two gold particles randomly distributed throughout the cell. Clusters of two to three gold particles were observed in only about 1% of the cells. In these cases, the gold particles were always next to the central plaque, at the half bridge of the SPB. The gold particles were directed toward the cytoplasmic side of the nuclear envelope (Fig. 5, J-L) suggesting that

Karlp is associated with the cytoplasmic side of the half bridge.

Cdc31p Interacts with Karlp and Nuf1p/Spcl10p

Mutants of *karl* (Rose and Fink, 1987) and *cdc31* (Byers, 1981a,b) have similar phenotypes; they arrest as large budded cells with a single, enlarged SPB lacking a satellite. This finding suggests that the two proteins act along the same pathway in SPB duplication, and may even interact. However, a direct interaction between Cdc31p and Karlp seemed unlikely, since the two proteins have been localized to different substructures of the SPB (Vallen et al., 1992b; Spang et al., 1993). Our current studies, which indicate that Karlp and Cdc31p are associated with the half bridge of the SPB, modify this picture.

Cdc31p shows a striking homology to CaM (Baum et al., 1986). The interaction of CaM with other proteins was investigated by the so-called overlay assay (Glenney and Weber, 1980). Radiolabeled CaM was incubated with filter-immobilized proteins. The interaction was then assayed by autoradiography. To identify proteins which interact with Cdc31p, we performed an overlay assay with [³⁵S]Cdc31p. Cdc31p was selectively labeled by the T7 system (Tabor and Richardson, 1985), then purified as described in Materials and Methods. [³⁵S]Cdc31p was incubated with proteins from a total yeast extract and isolated nuclei (Fig. 6 A). One protein interacting with [³⁵S]Cdc31p had a molecular mass of 53 kD; suggesting that it might be Karlp. To test whether [³⁵S]Cdc31p binds to recombinant Karlp, we expressed *GST-KARI* and truncations of *GST-KARI* in *E. coli*. Recently, it was reported that CaM binds to the COOH terminus of Nuf1p/Spcl10p (Geiser et al., 1993). To test whether Cdc31p interacts with Nuf1p/Spcl10p, we also expressed the NH₂- and COOH-terminal part of Nuf1p/Spcl10p as fusion proteins with GST in *E. coli* (*GST-N-NUF1* and *GST-C-NUF1*). Fig. 6 B shows a Coomassie blue stained gel of the *E. coli* extracts containing the GST-Karlp and GST-Nuf1p fusion proteins.

E. coli extracts with the various GST-Karlp and GST-Nuf1p fusion proteins (Fig. 6 B) were transferred to nitrocellulose membranes (Fig. 6, C and D). After blocking the protein binding sites of the membranes, the blots were incubated with [³⁵S]Cdc31p (Fig. 6 C) or [³⁵S]CaM (Fig. 6 D) in the presence (+Ca²⁺) and absence (+EGTA) of Ca²⁺. CaM from *Saccharomyces cerevisiae* was used in parallel to Cdc31p as a control. Free Ca²⁺ was removed by the chelator EGTA in one part of the experiment to determine whether the binding specificity of Cdc31p and CaM is Ca²⁺-dependent. Cdc31p bound to GST-Karlp and GST-ΔKarlp but not to GST-ΔΔKarlp (a truncation of the COOH-terminal half of Karlp) in the presence of Ca²⁺ (Fig. 6 C, +Ca²⁺). In addition, an interaction was detected with the C-terminal (GST-C-Nuf1p), but not the NH₂-terminal, part of Nuf1p/Spcl10p (GST-N-Nuf1p). CaM specifically interacted with the COOH-terminal part of Nuf1p/Spcl10p in the presence of Ca²⁺ but interacted neither with the NH₂-terminal part of Nuf1p/Spcl10p nor with the different GST-Karlp constructs (Fig. 6 D, +Ca²⁺). As previously described (Geiser et al., 1993), the interaction of CaM with GST-C-Nuf1p was not observed in the absence of Ca²⁺ (Fig. 6 D, +EGTA). Cdc31p bound to more proteins in the absence of Ca²⁺ (Fig. 6 C, +EGTA). The GST-Karlp constructs,

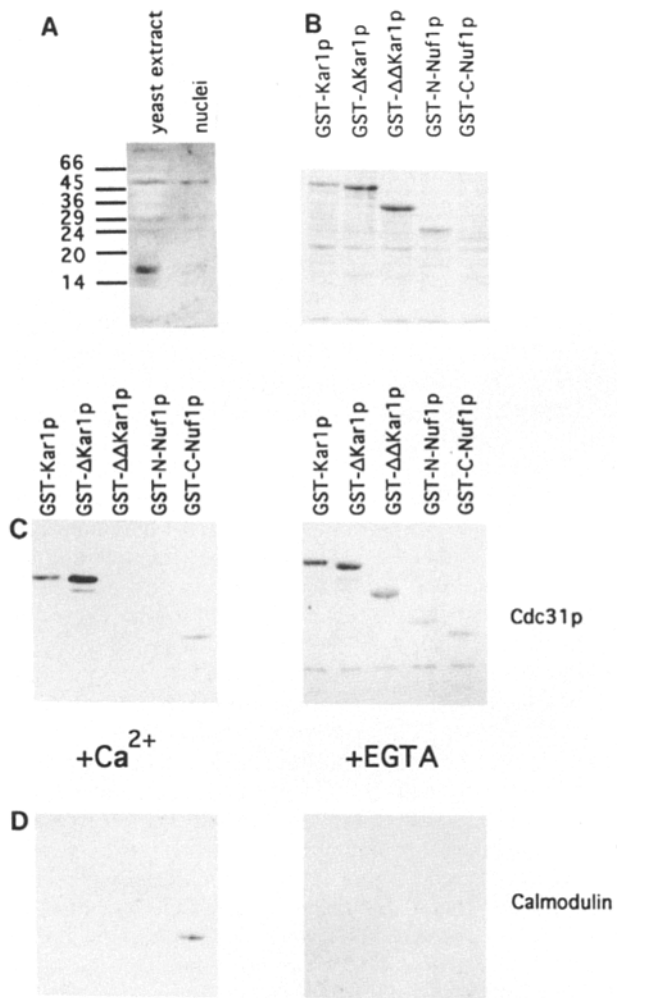


Figure 6. Cdc31p interacts with Karlp and Nuf1p/Spcl10p. (A) A total yeast extract or isolated nuclei of strain BJ5626 was separated on a 12% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated with [³⁵S]Cdc31p as described in Materials and Methods. (B) GST-Karlp and GST-Nuf1p/Spcl10p fusion proteins expressed in *E. coli* BL21 (DE3). The gel was stained with Coomassie blue. (C and D) Overlay assays of samples shown in B using (C) [³⁵S]Cdc31p or (D) [³⁵S]CaM in the presence of Ca²⁺ or EGTA. The dried membranes were exposed to autoradiography.

GST-N-Nuf1p and GST-C-Nuf1p and at least one *E. coli* protein were labeled by [³⁵S]Cdc31p when Ca²⁺ was removed by the chelator EGTA (Fig. 6 C, +EGTA). The Ca²⁺-free [³⁵S]Cdc31p did not bind to GST (data not shown), suggesting that Cdc31p interacted with the Karlp and Nuf1p portions of the GST-Karlp and GST-Nuf1p fusion proteins in the absence of Ca²⁺.

Since Cdc31p interacts with GST-ΔKarlp but not GST-ΔΔKarlp (Fig. 6 C), Cdc31p must bind to the COOH-terminal half of Karlp. Further deletions in this region of Karlp were constructed to determine the binding site of Cdc31p (Fig. 7 A). GST-ΔKarlp, GST-ΔKarlp-Δ, GST-ΔKarlp-2Δ, GST-ΔKarlp-3Δ, GST-ΔKarlp-4Δ and GST were purified as described in Materials and Methods. GST-Karlp was not used in this experiment because the protein is insoluble in *E. coli*. Equal molar amounts of each fusion protein were

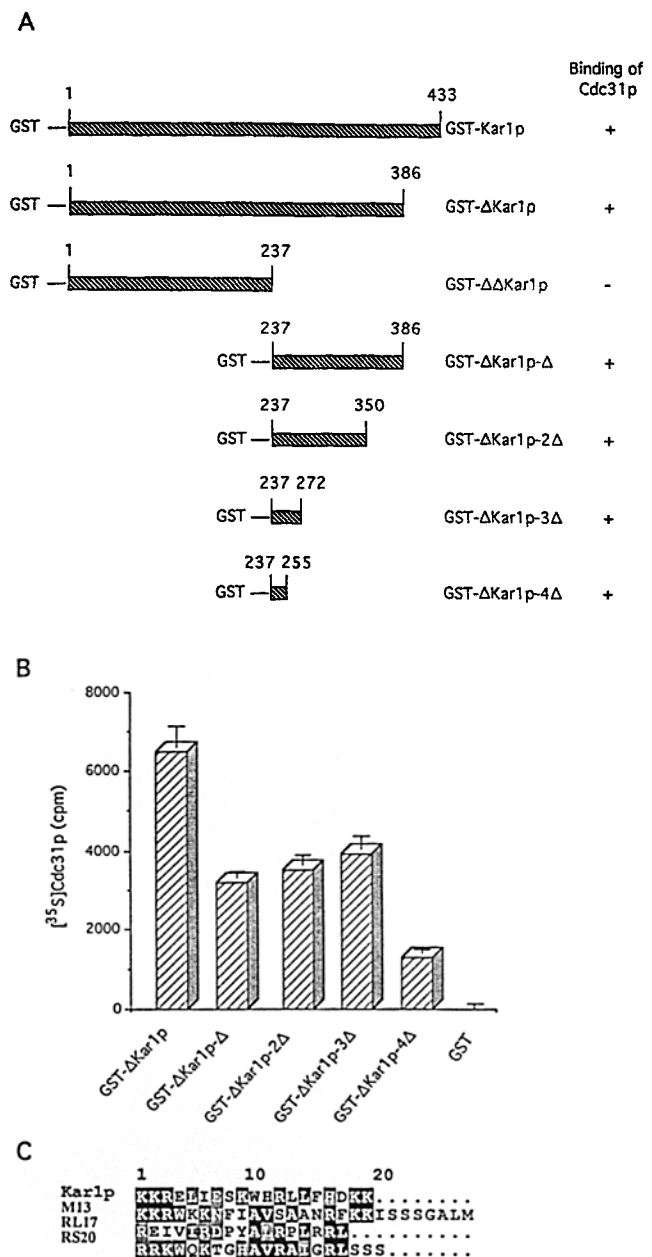


Figure 7. Identification of a Cdc31p-binding region in Karlp. (A) Various GST-KAR1 gene fusions were constructed as described in Materials and Methods using restriction sites in the KAR1 coding region. (B) Quantitation of Cdc31p-binding to Karlp-fragments. Purified GST-ΔKarlp, GST-ΔKarlp-Δ, GST-ΔKarlp-2Δ, GST-ΔKarlp-3Δ, GST-ΔKarlp-4Δ, and GST were blotted to a nitrocellulose membrane as described in Materials and Methods. The membrane was incubated with [³⁵S]Cdc31p. Sections of the membrane containing the fusion proteins and bound [³⁵S]Cdc31p were cut out. The radioactivity was determined by a liquid scintillation counter. The experiment was performed in triplicates. (C) The potential Cdc31p-binding peptide of Karlp (amino acids 239–257 of Karlp) in comparison to CaM-binding domains. M13: CaM binding peptide from rabbit skeletal muscle myosin light chain kinase (Blumenthal et al., 1985); RL17: Amino acid sequence of residues 329–345 of the γ subunit of rabbit skeletal muscle phosphorylase kinase (Reimann et al., 1984); RS20: High-affinity CaM binding peptide from chicken myosin light chain kinase (Lukas et al., 1986).

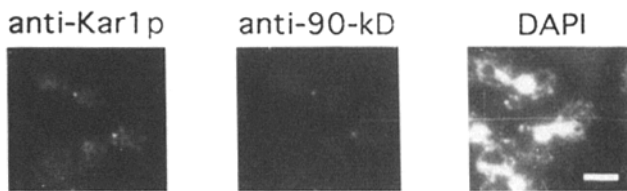


Figure 8. Kar1p is associated with the SPB in *cdc31* cells. Strain ESM56 was grown in SM Medium containing adenine, lysine, tryptophan and uracil. Cells were diluted with SC Medium containing 2 mM methionine to repress the expression of *CDC31*. The culture was incubated for 8 h at 30°C. No Cdc31p was detectable after this incubation period by immunoblotting (data not shown). ESM56 cells were then fixed and stained with affinity purified rabbit anti-Kar1p and monoclonal mouse anti-90-kD antibodies. DNA was stained with DAPI. Bar, 5 μ m.

blotted to a nitrocellulose membrane and then incubated with [³⁵S]Cdc31p. Pieces of nitrocellulose membrane containing the fusion proteins with the bound Cdc31p were excised and assayed for [³⁵S]Cdc31p. Two times the Cdc31p was bound to GST- Δ Kar1p than to GST- Δ Kar1p- Δ , GST- Δ Kar1p-2 Δ and GST- Δ Kar1p-3 Δ (Fig. 7 B). The binding efficiency decreased further when an additional 17 amino acids of GST- Δ Kar1p-3 Δ were deleted (GST- Δ Kar1p-4 Δ), suggesting that the Cdc31p-binding domain of Kar1p was in part removed in GST- Δ Kar1p-4 Δ . Since no binding of [³⁵S]-Cdc31p to GST was observed, the interaction with GST-Kar1p-4 Δ must depend on the Kar1p portion of the fusion protein. The folding of GST could be responsible for the lower binding efficiency of GST- Δ Kar1p- Δ , GST- Δ Kar1p-2 Δ and GST- Δ Kar1p-3 Δ compared to GST- Δ Kar1p. Therefore, we heat-denatured the various GST-Kar1p proteins with 0.2% SDS and bound the GST-Kar1p proteins under denaturing conditions to the nitrocellulose membrane. The Cdc31p-binding assay was then repeated. A very similar result was obtained (data not shown), indicating that the folding of GST-Kar1p proteins did not influence the binding of Cdc31p.

The comparison of the extended Cdc31p-binding region of Kar1p-4 Δ with CaM-binding peptides reveals a sequence with conserved features (Fig. 7 C) which, however, differs in several positions from the CaM binding sequences.

Kar1p is Associated with the SPB in the Absence of Cdc31p, though the Localization of Cdc31p with the SPB Is Dependent on Kar1p

We tested whether the interaction of Kar1p with the SPB is dependent on Cdc31p. *CDC31* of strain ESM56 is under control of the methionine-repressible *MET3* promoter (Albermann, K., U. Fackler, A. Spang, K. Grein, and E. Schiebel, manuscript in preparation). The wild-type *CDC31* of ESM56 is disrupted by the *HIS3* gene while *MET3-CDC31* is integrated into the *LEU2* locus (Table I). The addition of methionine to a culture of ESM56 causes the total depletion of Cdc31p followed by a cell cycle arrest. Eight hours after the addition of methionine to a culture of ESM56, Cdc31p was not detectable by immunoblotting with anti-Cdc31p antibodies in a cell lysate (data not shown). The localization of Kar1p in cells of ESM56 was investigated after depletion of Cdc31p by indirect immunofluorescence with anti-Kar1p antibodies. Kar1p was associated with the SPB of cell cycle ar-

Table III. SPB Staining of Yeast Cells

Strain	Number of SPB's	Stained SPB's
YPH499	16	8
ESM115	18	0

rested ESM56 cells (Fig. 8). An identical result was obtained with *cdc31-1* cells (Byers, 1981b) shifted to the non-permissive temperature (data not shown). Therefore, we concluded that Cdc31p is not required for the localization of Kar1p with the SPB. Alternatively, Cdc31p may play a direct role in localizing Kar1p to the SPB but then was lost due to turnover.

We also determined whether Cdc31p is associated with the SPB of *kar1* cells. A strain, ESM115, was constructed in which *KAR1* is under control of the *MET3* promoter. Eight hours after the addition of methionine to a culture of ESM115, cells arrested with a uniformly large bud and a single SPB. No Kar1p was detectable by immunoblotting in a cell extract of ESM115 after growth in methionine-containing medium (data not shown).

Cells of ESM115 were incubated in methionine-containing medium and then prepared for immunoelectron microscopy with anti-Cdc31p antibodies as described by Spang et al. (1993). As a control, the parent strain YPH499 grown under the same conditions was labeled in parallel. The SPB of cells of YPH499 were stained at the half bridge (Spang et al., 1993; Table III). In contrast to YPH499, no SPB staining was detected in cells of ESM115 (Table III). The levels of Cdc31p in lysates of ESM115 and YPH499 were approximately the same (as tested by immunoblotting), indicating that Cdc31p was not degraded in the absence of Kar1p (data not shown). This result suggests that binding of Cdc31p to the SPB requires either Kar1p or that the structure of the SPB of the *KAR1* mutant is changed in such a way that Cdc31p binding is eliminated. A similar result was reported by Biggins and Rose (1994), using the temperature sensitive strain MS2083 (*kar1- Δ 17*).

Discussion

Localization of the Kar1p Protein

Despite the important role of the centrosome in each phase of the life cycle of eukaryotic cells, relatively little is known about its molecular structure and cell cycle regulation. This is also valid for yeast *Saccharomyces cerevisiae*, where centrosomal functions are provided by the SPB (Byers, 1981a, b). For example, the sublocalization of only three SPB proteins have been reported to date; Nuf1p/Spc110p, 90-kD protein and Cdc31p (Kilmartin et al., 1993; Rout and Kilmartin, 1990; Spang et al., 1993). The location of these proteins within the SPB is outlined in Fig. 9 A.

Kar1p is also a potential SPB component. Kar1p functions in karyogamy of mating yeast cells as well as in SPB duplication during mitosis (Conde and Fink, 1976; Rose and Fink, 1987). Since Kar1p is essential, it is important to establish its subcellular localization. Kar1p- β -galactosidase fusion proteins were associated with the SPB in a cell cycle-dependent manner. Furthermore, one of these hybrid proteins localized to the outer plaque of the SPB (Vallen et al.,

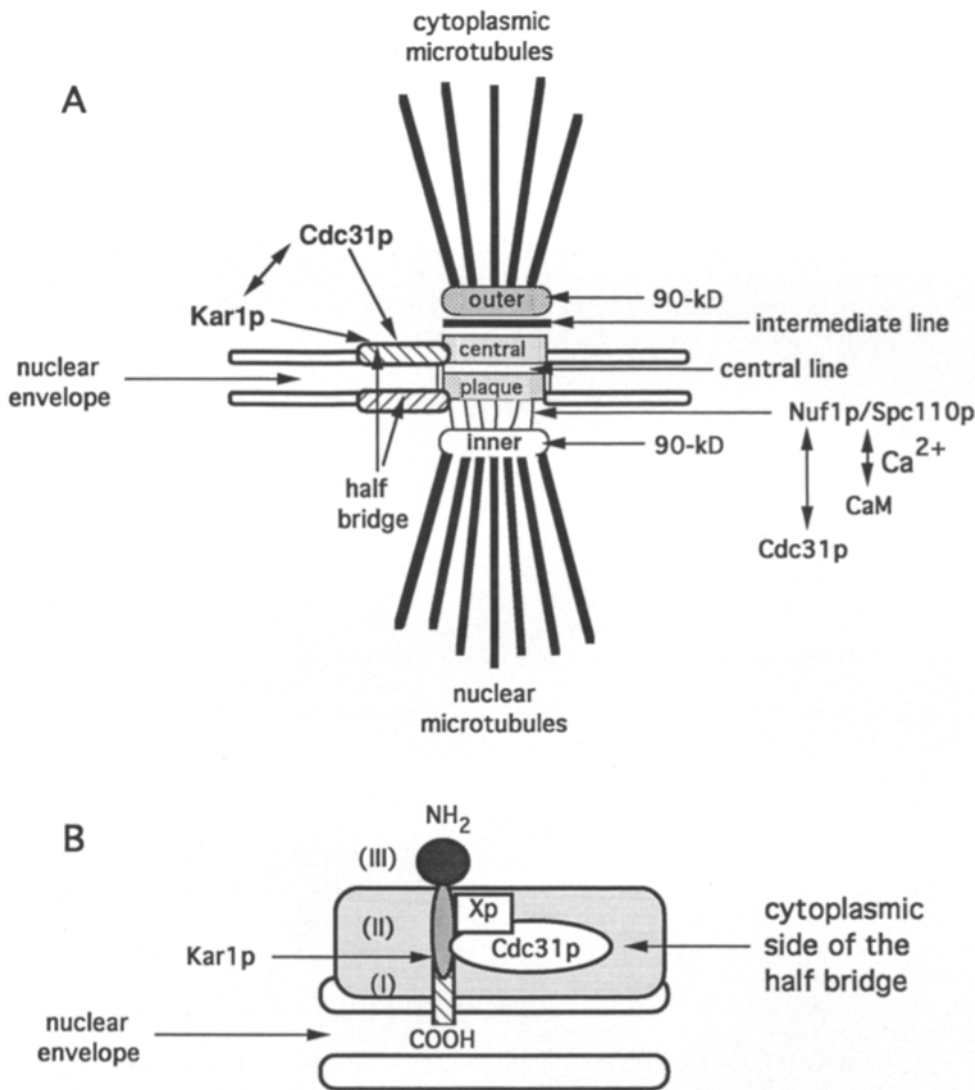


Figure 9. (A) Schematic diagram showing the localization of Karlp and Cdc31p relative to that of known SPB components. (B) A model for the interaction of Cdc31p and Karlp with the unidentified protein Xp at the cytoplasmic side of the half bridge. For a description see text.

1992b). Lately, the question has been raised whether the behavior of the Karlp- β -galactosidase fusion proteins reflects that of wild-type Karlp (Biggins and Rose, 1994). In this paper, we now show that Karlp behaves quite differently from the Karlp- β -galactosidase fusion proteins. We demonstrate for the first time that Karlp is a permanent component of the half bridge of the SPB. Taken together, four lines of evidence support our conclusion: (a) Karlp cofractionated with a highly enriched preparation of SPBs (Fig. 4). (b) Karlp was associated with the SPB during the entire cell cycle, as revealed by indirect immunofluorescence (Figs. 2 and 3). (c) The half bridge or bridge of isolated SPBs was seen by immunoelectron microscopy to be labeled by affinity purified antibodies (Fig. 5, A-E). (d) Epitope tagged, functional Karlp was also associated with the half bridge as shown by immunoelectron microscopy with 12CA5 monoclonal antibodies using whole yeast cells (Fig. 5, J-L) and isolated SPBs (Fig. 5, F-I).

Because the gold particles of the immunoelectron microscopy were directed toward the cytoplasmic side of the nuclear envelope (Fig. 5, D and J-L), Karlp may be associated with the cytoplasmic side of the half bridge or the bridge substructure of the SPB (Fig. 9 A). This suggests that the half

bridge is comprised of two subcompartments, one nuclear and the other cytoplasmic, which differ in composition. At least two domains of Karlp are necessary for its localization with the half bridge. The COOH-terminal membrane anchor targets Karlp- β -galactosidase fusion proteins to the nuclear envelope (Fig. 9 B [I]; Vallen et al., 1992b). The center of Karlp (amino acids 190-259) can direct Karlp- β -galactosidase fusion proteins to the SPB (Fig. 9 B [II]). Karlp fusion proteins containing only the central domain are mistargeted to the outer plaque of the SPB (Vallen et al., 1992b). The NH₂-terminus of Karlp (Fig. 9 B [III]) has functions in karyogamy (Vallen et al., 1992a) and is most likely exposed to the cytoplasm. Our working model is that Karlp is at first directed to the cytoplasmic side of the nuclear envelope through its hydrophobic membrane anchor. In a second step, Karlp might then be targeted to the half bridge of the SPB by its interaction with at least one SPB component (Fig. 9 B; Xp). Whether this component is Cdc31p is not clear. The observation that Karlp is associated with the SPB in the absence of Cdc31p (Fig. 8) argues against a role of Cdc31p in directing Karlp to the SPB. However, Cdc31p may play a direct role in localizing Karlp to the SPB but then was lost after shifting the *cdc31* cells to non-permissive conditions.

Wild-type Karlp is associated with the SPB during the entire yeast cell cycle (Fig. 3), though the Karlp- β -galactosidase fusion proteins are not. We suggest that Karlp is a permanent component of the half bridge or bridge of the SPB. The Karlp was always seen by immunoelectron microscopy to be with the half bridge or bridge of the SPBs isolated from an unsynchronized, logarithmic growing yeast. Since the Karlp- β -galactosidase fusion proteins are mistargeted to the outer plaque of the SPB, it is not surprising that they differ from wild-type Karlp in their cell cycle behavior.

Interaction of Cdc31p with Karlp

Mutants of both *KARI* (Rose and Fink, 1987) and *CDC31* (Byers, 1981a,b) exhibit a similar phenotype; they arrest as budded cells with a single, enlarged SPB lacking a satellite. This finding suggests that the two proteins act in the same pathway in SPB duplication. A direct interaction of Karlp and Cdc31p seemed unlikely, since Karlp- β -galactosidase and Cdc31p were localized to different substructures of the SPB complex. From our studies, it is now clear that both Karlp and Cdc31p are components of the cytoplasmic side of the half bridge (Spang et al., 1993, Fig. 5). We therefore used an in vitro assay which has been successfully used to study the interaction of CaM with target proteins (Glenney and Weber, 1980) to investigate whether Cdc31p and Karlp directly interact. Our findings clearly indicate that Cdc31p interacts in vitro with the COOH-terminal half of Karlp (Figs. 6 and 7). In addition, Cdc31p binds to the COOH terminus of Nuflp/Spcl10p, which contains a binding site for CaM (Geiser et al., 1993). Whether Cdc31p binds to the CaM-binding site of Nuflp/Spcl10p or whether the latter protein contains an additional Cdc31p-binding motif remains to be determined. The significance of this interaction is not clear, especially because Cdc31p and Nuflp/Spcl10p are associated with different substructures of the SPB (Fig. 9 A). However, CaM did not bind to Karlp, indicating a different substrate specificity of the two Ca²⁺-binding proteins. The Ca²⁺ dependence of the interaction of CaM and Cdc31p with substrates was also different. While CaM did not bind to any protein in the absence of Ca²⁺ (Fig. 6 D, Geiser et al., 1993), Cdc31p interacted with additional proteins (one *E. coli* protein, NH₂ terminus of Nuflp/Spcl10p and Karlp, Abb. 6C) when Ca²⁺ was removed by EGTA. Although we have not studied the Ca²⁺-independent interactions of Cdc31p further, our observations suggest that the binding specificity of Cdc31p is regulated by Ca²⁺. The proteins that interacted with Cdc31p in the absence of Ca²⁺ may have binding sites which are only recognized by Ca²⁺-free Cdc31p.

We have localized the core of one Cdc31p-binding site of Karlp to amino acids 237 to 255 (Fig. 7, A and B) which is indicated by the binding of Cdc31p to GST- Δ Karl-4 Δ . The total Cdc31p-binding region of Karlp is probably larger than the 18 amino acids of Δ Karl-4 Δ . This is suggested by the observation that NH₂-terminal (Δ Karl) and COOH-terminal extensions (Δ Karl- Δ , Δ Karl-2 Δ and Δ Karl-3 Δ) of the Δ Karl-4 Δ peptide increase the efficiency of Cdc31p-binding (Fig. 7 B). We do not think that the folding of GST inhibits the interaction of Cdc31p with Karlp, since unfolding of GST by SDS did not influence the binding of Cdc31p to the GST-Karlp constructs.

We compared amino acids 230–260 of Karlp with published CaM-binding peptides. Amino acids 239–257 of Karlp had several features in common with the CaM-binding peptides (Fig. 7 C). The positively charged amino acids at positions 1, 2, 3, 12, 16, and the hydrophobic amino acids at positions 13 and 14 were also present in 2 of the 3 CaM-binding peptides (Fig. 7 C). The acidic amino acid in position 7 and the basic amino acids in positions 9, 18, and 19 coincided with one of the three CaM-binding peptides. In contrast to the CaM-binding peptides, the potential Cdc31p-binding site contained three acidic amino acids which are not found in CaM-binding motifs and might determine the specificity of the interaction with Cdc31p. Furthermore, computer analysis of this potential Cdc31p-binding peptide by the method of Rost et al. (1993) predicted a high probability of alpha-helical conformation. This is also the conformation of CaM-binding peptides (O'Neil et al., 1987).

While this manuscript was in preparation, Biggins and Rose (1994) reported the interaction of Cdc31p with Karlp using a very similar approach. The Cdc31p-binding domain was localized to amino acids 187–299 of Karlp including the portion of Karlp which we mapped as Cdc31p-binding region (amino acids 237–255). It remains unclear why Karlp- β -galactosidase fusion proteins encoded by plasmids pMR404 (amino acids 1–259 of Karlp) and pMR1850 (amino acids 187–259) did not interact with Cdc31p (Biggins and Rose, 1994). These fusion proteins were expressed in yeast and enriched by immunoprecipitation. Possibly, the fusion site with β -galactosidase at the COOH terminus of the Cdc31p-binding motif interferes with Cdc31p-binding. Further, Biggins and Rose (1994) did not observe a Ca²⁺-dependence of Cdc31p-binding, although they reported an increase in background binding when EGTA was present. This discrepancy might be explained by different methods used for the labeling of Cdc31p in the two studies. We specifically labeled Cdc31p in vivo using the T7 system, and then purified the ³⁵S-labeled protein. Biggins and Rose (1994) labeled purified Cdc31p with a sulphur labeling kit that adds ³⁵S to free amino groups and therefore modifies Cdc31p.

The question remains, whether Cdc31p and Karlp interact in vivo. We have not been able to demonstrate the interaction of Karlp and Cdc31p by immunoprecipitation with anti-Cdc31p or anti-Karlp antibodies (A. Spang, unpublished observations). The reasons for this might be twofold. The complex containing Karlp and Cdc31p may be disrupted during the extraction of SPBs. Alternatively Karlp and Cdc31p may interact only transiently during a short period of the cell cycle. Karlp has a potential p34^{cdc28} phosphorylation site (TPTK; Moreno and Nurse, 1990) at amino acids 294–297 which could be involved in such a regulation. Genetic studies by Vallen et al. (1994) identified *CDC31* as a high copy number and ectrogenic suppressor of a temperature sensitive mutation of *KARI*. While this suggests an interaction of Cdc31p and Karlp, some *cdc31* alleles suppressed a complete deletion of *KARI* (Vallen et al., 1994), favoring a bypass mechanism. In summary, considering the colocalization of Karlp and Cdc31p within the same substructure of the SPB (Fig. 5; Spang et al., 1993), the in vitro interaction of Cdc31p and Karlp (Figs. 7 and 8; Biggins and Rose, 1994) and the genetic interaction between *CDC31* and *KARI* (Vallen et al., 1994) it is most likely that Cdc31p and Karlp interact at least transiently in vivo.

Interestingly, amino acids 235 to 294 of Karlp can be deleted without affecting the viability of yeast cells, while a deletion of amino acids 191–246 caused a temperature sensitive phenotype (Vallen et al., 1992a). These deletions interfere with the minimal Cdc31p-binding site which we have identified (amino acids 237–255; Fig. 7 A) and with the more extended region of Biggins and Rose (1994; amino acids 190–299 of Karlp). This finding raises the question, such as whether the Cdc31p-binding site(s) of Karlp fulfill an essential function?

Vallen et al. (1994) proposed a model in which Karlp helps to localize Cdc31p to the SPB and the Cdc31p then initiates SPB duplication via interaction with a downstream effector. We favor a modified version of this model in which Cdc31p and Karlp are permanent components of the cytoplasmic side of the half bridge of the SPB (Spang et al., 1993; Figs. 2 and 3). Cdc31p and Karlp may form a complex with at least one additional protein Xp (Fig. 9 B). Xp has binding sites for both Karlp and Cdc31p. A potential candidate for Xp is a 30-kD protein which has been demonstrated by cross-linking studies to be next to Cdc31p (A. Spang, unpublished observations). Signals from Cdc31p and Karlp would be transmitted to Xp, which then initiates formation of the satellite in early G₁ phase of the cell cycle. The Karlp signal could be the dephosphorylation of Karlp at the end of mitosis. Cdc31p, on the other hand, could sense local changes in Ca²⁺ concentration. We assume that the signals transmitted to Xp by Karlp and Cdc31p are in some way redundant. However, it is possible that Karlp and Cdc31p may be active during different phases of the cell cycle. Mutations in *CDC31* could result in a Cdc31p* which is activated earlier in the cell cycle, perhaps by an altered binding constant for Ca²⁺, and therefore fully overlaps with the Karlp signal. This would explain why certain mutations in *CDC31* can substitute for deletions in *KAR1*. In summary, we consider the Cdc31p/Karlp/Xp complex to be a key regulator in SPB duplication which may transmit cell cycle-specific signals to yet unknown components of the SPB.

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