Spc42p: A Phosphorylated Component of the *S. cerevisiae* Spindle Pole Body (SPB) With an Essential Function During SPB Duplication

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Abstract. The 42-kD component of the *S. cerevisiae* spindle pole body (SPB) localizes to the electron-dense central plaque of the SPB (Rout, M.P., and J.V. Kilmartin. 1991. *Cold Spring Harbor Symp. Quant. Biol.* 56:687–691). We have cloned the corresponding gene *SPC42* (spindle pole component) and show that it is essential. Seven temperature-sensitive (ts) mutants in *SPC42* were prepared by error-prone PCR. We found that a change to a proline residue in a potential coiled-coil region of Spc42p was responsible for the ts phenotype in at least three alleles, suggesting that formation of the coiled-coil is essential for normal function. The mutant cells showed a phenotype of predominantly single or bilobed SPBs often with an accumulation of unstructured electron-dense material associated with the bridge structure adjacent to the SPB. This phenotype suggests a defect in SPB duplication. This was confirmed by examining synchronized mutant cells that lose viability when SPB duplication is attempted. Spc42p is a phosphoprotein which shows some cell cycle-regulated phosphorylation. Overexpression of Spc42p causes the formation of a disc- or dome-shaped polymer composed of phosphorylated Spc42p, which is attached to the central plaque and associated with the outer nuclear membrane. Taken together, these data suggest that Spc42p forms a polymeric layer at the periphery of the SPB central plaque which has an essential function during SPB duplication and may facilitate attachment of the SPB to the nuclear membrane.

The spindle pole body (SPB)[1] of *Saccharomyces cerevisiae* is the functional equivalent of the centrosome in the yeast cell. It is a complex cylindrical multi-layered structure lying in the nuclear envelope. The central layer or plaque lies in the plane of the nuclear membrane. On the cytoplasmic side is the outer plaque that organizes cytoplasmic microtubules, while nuclear or spindle microtubules are organized from a more ill-defined inner plaque (Byers, 1981*a*; Rose et al., 1993; Winey and Byers, 1993). Like the centrosome, the SPB is duplicated during G_1/S. The daughter SPB assembles on the satellite structure, which is cytoplasmic and attached to the mother SPB by a short electron-dense segment of the nuclear membrane called the half-bridge (Byers and Goetsch, 1975; Winey et al., 1991*a*). During or after duplication the daughter SPB is inserted into the nuclear envelope. Both SPBs remain connected by the bridge until this structure is somehow partitioned between the two SPBs as they migrate to opposite positions on the nuclear envelope and form the mitotic spindle (Byers and Goetsch, 1974).

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1. *Abbreviations used in this paper:* CIP, calf intestinal alkaline phosphatase; MCB, MluI cell cycle box; SPB, spindle pole body; SPC, spindle pole component; ts, temperature-sensitive.

At present an explanation of SPB functions in molecular terms is in its very early stages. Genetic and biochemical approaches have identified a number of structural components of the SPB. Cdc31p (Baum et al., 1986; Spang et al., 1993), Kar1p (Rose and Fink, 1987; Spang et al., 1995), and calmodulin (Geiser et al., 1993; Stirling et al., 1994) were identified from genetic screens, while 110-, 90-, and 42-kD components were identified using mAbs prepared against an enriched SPB preparation (Rout and Kilmartin, 1990, 1991). Screens for mutants with defects in SPB assembly have identified *MPS1*, *MPS2* (Winey et al., 1991*b*), and *NDC1* (Thomas and Botstein, 1986; Winey et al., 1993), although it has not yet been established what precise role these gene products have in SPB duplication.

A combination of genetic and biochemical experiments has provided a partial understanding of the functions of some of these SPB components. Cdc31p and Kar1p are both essential proteins required for SPB duplication. They interact with each other (Biggins and Rose, 1994; Vallen et al., 1994; Spang et al., 1995) and temperature-sensitive (ts) mutants in both proteins show the same phenotype: cells arrest with a single enlarged SPB (Byers, 1981*b*; Rose and Fink, 1987). SPB components in these cells appear to assemble around the preexisting SPB rather than the satellite, perhaps due to a defect in the associated half-bridge, since this is where both Cdc31p and Kar1p are localized (Spang et al., 1993; Spang et al., 1995). Cdc31p is a calmod-

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ulin-like protein (Baum et al., 1986) that has homologues in *Chlamydomonas* (Huang et al., 1988) and mammals (Lee and Huang, 1993; Errabolu et al., 1994). Kar1p is a membrane protein (Rose and Fink, 1987) with no homologues yet known in higher eukaryotes. The gene for the 110-kD SPB component, *SPC110* (Kilmartin et al., 1993) is identical to *NUF1* (Mirzayan et al., 1992). Spc110p contains a long central coiled-coil region. Deletions in this coiled-coil show that Spc110p acts as a spacer protein between the central plaque and the ends of the nuclear microtubules (Kilmartin et al., 1993). Spc110p is a calmodulin-binding protein and the essential function of calmodulin during mitosis occurs via an interaction with Spc110p (Geiser et al., 1993; Stirling et al., 1994).

While some progress has been made in understanding the molecular architecture of the SPB, very little is known about the regulation of its assembly at G_1/S. The CDC28/cdc2 kinase clearly has a pivotal role in both daughter SPB assembly and the initiation of S phase (Hartwell, 1974; Nurse, 1990). However in budding yeast few potential substrates of the CDC28 kinase have been identified. The kinase Mps1p (Lauze et al., 1995) may also be involved in SPB duplication but its substrates are also unknown. In this paper we show that the 42-kD component, which localizes to the electron-dense central plaque of the SPB (Rout and Kilmartin, 1991), is a phosphoprotein showing some cell cycle-regulated phosphorylation. Its gene, *SPC42* (Spindle Pole Component), is essential and ts mutants show alterations in the central plaque structure and defects in SPB duplication. Experiments on synchronized cells suggest that *SPC42* exercises an essential function during SPB duplication. Overexpression of Spc42p causes the formation of a polymer associated with the central plaque which is composed of phosphorylated Spc42p.

Materials and Methods

Cloning of SPC42

Cell culture supernatants from the pooled anti–42-kD mAbs (Rout and Kilmartin, 1991) were used to screen 10^6 phage from a yeast genomic DNA expression library in λgt11 (a gift of A. Sugino, Dept. Molecular Immunology, Osaka University, Japan). Seven positives were obtained and plaque purified. Restriction mapping with EcoRI grouped them into two classes represented by phages 305 and 306 (see Fig. 2). A larger insert, the 5.5-kb Sau3AI fragment (see Fig. 2), was obtained by using the phage insert to screen a plasmid library (Hardwick et al., 1992). A 3.6-kb XmnI fragment was sequenced on both strands using the ExoIII deletion method (Henikoff, 1984) with suitable oligonucleotide primers to cover gaps. Base pair numbers start from the A of the first methionine of the open reading frame.

Three fragments of *SPC42* were expressed in *Escherichia coli* using a modified T7 expression vector pMW172 (Way et al., 1990). For T7-2, a BstYI-EcoRV fragment (bases 8–666) was used to express residues 3–222, for T7-3 an NcoI-blunted ClaI fragment (bases 429–1087) was used to express residues 144–363 and for T7-4 a blunted KpnI-BstUI fragment (bases 155–969) was used to express residues 53–323. All ligations involving blunted sites were checked by sequencing and found to be correct. The EcoRV site at 666 used to construct T7-2 was present in the phage inserts but absent in the plasmid library insert due to a polymorphism. Two of the constructs, T7-2 and T7-4, produced fragments close to the expected sizes of 25.8 and 32.5 kD, respectively, but T7-3 (expected size 25.7 kD) migrated at an apparent size of 31 kD (Fig. 2 *b*). T7-2 was purified and used to prepare affinity-purified polyclonal mouse and rabbit antibodies (Goh and Kilmartin, 1993).

A disruption of *SPC42* was prepared by the one-step gene replacement method (Rothstein, 1983). The construct for this contained the *LEU2* gene placed between bases 2 and 969 of *SPC42* by a three-way ligation. A linear fragment was made by cutting with BamHI at −1012 and 2669 and this was transformed into the diploid yeast strain K842 to give AY1. K842 is an isogenic diploid of K699 (Nasmyth et al., 1990). All manipulations were carried out in these two strains. Southern blotting confirmed that the correct integration event had occurred (data not shown). After sporulation 30 tetrads were examined and all produced two viable (all Leu−) and two inviable spores. The inviable spores all germinated producing a cluster of 4–8 cells or buds. To check that the inviability was due to the loss of *SPC42*, AY1 was transformed with an XmnI-blunted NdeI fragment (−912–1692) of *SPC42* in the *CEN URA3* vector pRS316 (Sikorski and Hieter, 1989). After sporulation of this strain some tetrads now gave four viable spores, two of which were Leu+ and Ura+, showing that spores containing the disrupted *SPC42* gene were rescued by the plasmid, one of the progeny was retained as AY4. These progeny were dependent on the plasmid for growth since selection against the *URA3* plasmid on fluoroorotic acid medium (Boeke et al., 1984) gave no colonies.

Mutagenesis of SPC42

Mutagenesis of *SPC42* was carried out by PCR amplification (Foreman and Davis, 1993) with the dATP concentration reduced to 40 μM (Amon et al., 1993). Two sets of primers were used. Forward primers containing EcoRI sites hybridized to bases −555 to −533 and −528 to −510. Reverse primers containing BamHI sites hybridized to bases 1349 to 1368 and 1324 to 1342. PCR fragments were digested with EcoRI and BamHI and inserted into the *CEN TRP1* vector pRS314 (Sikorski and Hieter, 1989). This plasmid pool was transformed into AY4 and the cells plated onto −trp medium. This gave 40,000 transformants that were then replica plated onto −trp/fluoroorotic acid medium at 23 and 37°C. About 10% of the cells failed to grow at both temperatures, and seven ts mutants (see Fig. 4) were isolated where the ts phenotype was plasmid dependent. The open reading frames of *SPC42* in the seven alleles were sequenced with suitable oligonucleotide primers and the following amino acid changes found: for *spc42-1* the changes were M1:V, N58:Y, E71:G, L121:P, R163:G, D299:G, and M351:R. For *spc42-2*, the changes were I83:T, K87:E, N111:S, N129:D, S195:T, and S319:P. For *spc42-3* the changes were F74:S, L86:P, K88:R, and N170:D. For *spc42-4* the changes were I89:T, L112:P, T173:A, K176:R, Q320:H, N343:H, and R344:S. For *spc42-5* the changes were N39:K, Y55:H, Q110:P, T173:A, and I222:V. For *spc42-6* the changes were E71:G, S95:P, Q110:L, A167:G, L223:S, K276:R, and T359:I. For *spc42-7* the changes were M34:T, I62:V, S95:P, V261:G, K303:R, and N337:I. Up to three silent base changes were also found in each of the alleles. To discover which amino acid changes might be causing the ts phenotype BstYI-NcoI fragments (bases 8–429) and NcoI-ClaI fragments (bases 429–1087) were prepared from the mutagenized plasmids. These fragments were used in three-way ligations to prepare constructs to replace the corresponding sequences in *SPC42*. For *spc42-1*, *spc42-3*, and *spc42-5* individual mutations were prepared by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) within the BstYI-NcoI fragment and mutations transferred to *SPC42* as described above. These constructs, including *SPC42*, were transferred to the *TRP1* integrating vector pRS304 (Sikorski and Hieter, 1989) and a single cut made with SauI so that integration in AY4 was directed to the *trp1* locus. Fluoroorotic acid selection was used to remove the *SPC42* gene on the *URA* plasmid and Southern blotting used to confirm that the correct single integration event had occurred.

Most of the characterization of the phenotype was done on two alleles that had both low reversion frequencies (<1 in 10,000) and single copy integrants, *spc42-10* (Q110:P) and *spc42-11* (N58:Y, E71:G and L121:P). Both alleles are recessive, were rescued by transformation of *SPC42* on a *CEN* plasmid, and in crosses showed the segregation patterns expected of one lethal mutation (the disruption) and one ts mutation.

Overexpression of SPC42

To overexpress *SPC42* the wild-type promoter region between the MluI site at −232 and the BstYI site at the presumed start of the gene was replaced by bases 84–819 of the GAL1,10 promoter (Johnston and Davis, 1984). This promoter was amplified from plasmid YIp56X (Pelham et al., 1988) using GCCACGCGTCCACCTGTAACCAAAACA as forward primer and CCGGAGATCCGTTCAAGTCCTCTTCAGAAATGAGCTTTTGCTCATGCATTATAGTTTTTTCTCCTTG as reverse primer. The reverse primer was designed to fuse the myc epitope (Evan et al., 1985) to the presumed NH_2 terminus of Spc42p (Purnelle et al., 1993) and included an NsiI site in the initiator ATG codon to allow removal of the tagged construct to check its function. This construct should replace the initiator

methionine of Spc42p with the sequence MHEQKLISEEDL. The PCR product was digested with MluI and BstYI and ligated via a three-way ligation between the MluI site at −232 and the BstYI site at 7 of *SPC42* in pRS304 to give pJK404. This construct was cut with SauI to direct integration to the *trp1* locus of K699. A transformant (JKY813c) with 2–3 copies integrated at the *trp1* locus was selected to allow rapid induction of myc-tagged Spc42p. To check that the myc-tagged Spc42p was functional the *GAL* promoter in pJK404 was removed by digestion with MluI and NsiI and replaced by the wild-type promoter region amplified with the appropriate primers. This construct was cut with SauI, integrated at the *trp1* locus of AY4 and Ura^- Trp^+ transformants were selected. These had spontaneously lost the wild-type *SPC42* gene on the *URA3* plasmid and were now maintained by the myc-tagged *SPC42* in the integrated *TRP1* vector. Southern blotting was used to confirm that a single copy of *SPC42* had integrated at the *trp1* locus. This yeast strain grew normally showing that the myc-tagged Spc42p was functional, and with 9E10 anti-myc mAbs gave weak but recognizable immunofluorescent staining of SPBs superimposed on the background staining (data not shown).

Preparation of Spindle Pole Bodies and Heparin Extraction

JKY813c cells were grown overnight in 9 l of 2% raffinose, 2% bactopeptone, 1% yeast extract at 30°C, uninduced cells were harvested at 3×10^7 cells/ml. Induced cells were grown to 2×10^7 then 2% galactose was added for 2.5 h. Cells were washed once with water then treated with 700 ml 0.1 M Tris-Cl, pH 9.0, 10 mM EDTA, 1% mercaptoethanol (Byers and Goetsch, 1991) for 10 min at 22°C. Cells were washed once with water and twice with 1.2 M sorbitol, then diluted to 2×10^9 cells/ml and 2% galactose or raffinose added as appropriate. Cells were digested for 2.5 h at 30°C with 0.1 ml Glusulase (Dupont Corp., Boston, MA), 13 µl 10 mg/ml Zymolyase 20T (Seikagaku Corp., Tokyo, Japan), 2.6 µl 50 mg/ml Mutanase SP-299 (Novo Industri, Copenhagen, Denmark)/ml. Cells were harvested and lysed as described for BJ2168 in Rout and Kilmartin (1990). Care was taken to ensure complete resuspension of the crude nuclear pellet before the sucrose gradient. Nuclei were collected from the 2.0/2.1 M sucrose-PVP interface.

Pelleted nuclei were extracted (Rout and Kilmartin, 1990) with an increased amount of digitonin buffer (1 ml/55 OD 260 nm, note that the ratio in Rout and Kilmartin [1990] is in error, it should be 1 ml/ 100 OD 260 nm). The extracted nuclei were fractionated on sucrose gradients (Rout and Kilmartin, 1990) and SPBs were collected from the 2/2.25 M interface. SPBs were extracted with heparin in bt-DMSO (Rout and Kilmartin, 1990) but without mercaptoethanol, and fractionated on an expanded discontinuous sucrose gradient consisting of 1.3 ml of 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.5 M sucrose-bt containing 0.01% Tween (suggested by M. Rout). The tubes were spun for 40,000 rpm at 4°C for 4 h and heparin extracted material collected from the 2.25 M layer.

Alkaline Phosphatase Treatment

SPBs and the structures induced by overexpression of Spc42p were solubilized by addition of 0.4% SDS and boiling for 3 min. After cooling an equal volume of modified 2× RIPA buffer (0.3 M NaCl, 2 mM $MgCl_2$, 0.2 mM $ZnCl_2$, 4% NP-40, 1% sodium deoxycholate, 0.1 M Tris-Cl, pH 8.0) was added and the sample treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) (Carmo-Fonseca et al., 1993). Assays with *p*-nitrophenyl phosphate showed that the enzyme was fully active in this buffer. SDS gels were prepared with 18 × 20-cm glass plates and were 8–13% gradient gels.

Immunoprecipitation

Cells were grown to mid log phase then filtered, washed with cold water, and resuspended in an equal volume of cold water containing 1:50 solution P (Rout and Kilmartin, 1990). The cells were added to an equal volume of 2× Laemmli sample buffer (44% glycerol, 5.5% SDS, 0.067 M Tris-Cl, pH 6.8, 10% mercaptoethanol) in a boiling water bath, left for several minutes then one volume of glass beads was added, the solution reheated, and then whirl-mixed vigorously several times. One volume of 10× phosphatase inhibitors (0.5 M NaF, 10 mM sodium vanadate, 0.5 M β-glycerophosphate) and then 10 vol of modified RIPA buffer (without $MgCl_2$ or $ZnCl_2$) were added and the solution spun at 12,000 *g* for 3 min. All volumes are based on the resuspended cells volume. After preclearing by Sepharose–protein A beads (Pharmacia, London, UK) at 4°C, affinity-purified rabbit anti-Spc42p antibodies were added for 1 h at 4°C followed by Sepharose–protein A beads. After washing the beads were digested with alkaline phosphatase as above. For cells labeled with ^{35}S (Foiani et al., 1995) and ^{32}P (Rubin, 1975) the washed beads were digested with 20 µg/ml RNase A at 30°C for 15 min, washed again as above then boiled in SDS sample buffer in a different tube. The SDS gel bands resulting from immunoprecipitation always had a slightly less crisp appearance presumably due to a slight dephosphorylation during the immunoprecipitation. The inclusion of a cocktail of phosphatase inhibitors (50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 1 µM okadaic acid, and 10 nM microcystin) had no effect on this.

Immunofluorescent Staining of spc42-10 Cells with Anti–42-kD Antibodies

To maximize the immunofluorescent signal given by the anti–42-kD antibodies formaldehyde fixation was omitted. Instead cells were first spheroplasted then fixed in methanol and acetone (Rout and Kilmartin, 1990). After a 4-h block at 36°C *spc42-10* cells were washed with warm sorbitol then spheroplasted with 20 µg/ml Oxalyticase (Enzogenetics, Cornvallis, OR 97333) in 1 M Sorbitol/PBS, 0.14 M mercaptoethanol at 36°C for 30 min. Spheroplasts were washed with 1 M Sorbitol and allowed to recover in 1 M sorbitol, 2% glucose, 2% bactopeptone, 1% yeast extract for 20 min at 36°C before washing and fixation in methanol and acetone. Cells at 23°C were treated identically at 23°C except that the digestion was extended to 45 min. Cells were labeled with a mixture of the anti–42-kD mAbs and affinity-purified mouse polyclonal anti–42-kD antibodies to further maximize the signal.

Other Methods

Northern blots and immunofluorescence were carried out as described in Kilmartin et al. (1993). Cells were synchronized either by α-factor (Goh and Kilmartin, 1993) or by elutriation (Schwob and Nasmyth, 1993) at 10°C and 3,500 rpm. Different batches of elutriated cells formed buds at similar times, with between 54 and 83% small buds after 1.5 h at 36°C. Elutriated cells were treated with nocodazole at 15 µg/ml or hydroxyurea at 0.1 M and arrested as large-budded cells. Cell were fixed for flow cytometry as described by Nash et al. (1988). EM was as described by Byers and Goetsch (1991) with slight modifications (Goh and Kilmartin, 1993). The lead citrate stain (Reynolds, 1963) for thin sections of cells overexpressing Spc42p was diluted 10–100-fold with 0.01 M NaOH to prevent heavy precipitation of the lead onto the Spc42p polymer.

Results

Further Characterization of the Anti–42-kD mAbs

A preliminary characterization of the anti–42-kD mAbs, 70D1, 70E3, and 75A11, showed that they reacted specifically with a component of apparent molecular mass 42 kD in the enriched SPB preparations, which was localized to the central plaque of the SPB by immuno-EM (Rout and Kilmartin, 1991). In this paper we have further characterized these mAbs and show that under the fixation conditions used (Kilmartin et al., 1993), staining by immunofluorescence was confined to the region of the SPB (Fig. 1 *a*). There appeared to be a decrease in the intensity of SPB staining in post-anaphase cells. As with other SPB and spindle antigens the staining was very sensitive to formaldehyde fixation (Rout and Kilmartin, 1990). To examine the specificity of the mAbs further, we tested them on immunoblots of whole yeast cells but no signal was seen. The mAbs probably have lowered reactivity towards SDS-denatured antigens. A weak signal, which was specifically associated with the SPB-containing fraction (Fig. 1 *b*), appeared when detergent-extracted nuclei were fractionated on sucrose gradients (Rout and Kilmartin, 1990). This signal gained in intensity when the SPBs were further frac-

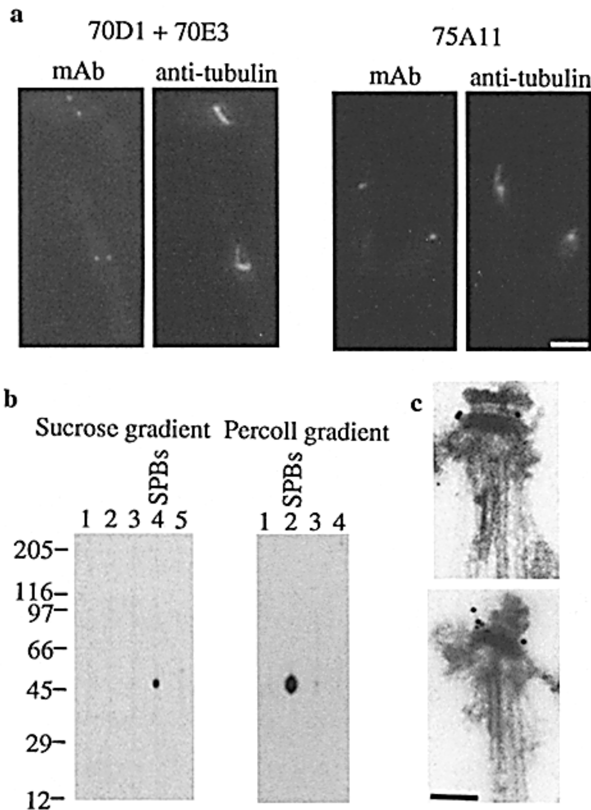


Figure 1. (*a*) Immunofluorescent staining of *S. uvarum* cells with the anti–42-kD mAbs 70D1 and 70E3 (*left*) and 75A11 (*right*). 70D1 and 70E3 were mixed because they probably react with the same epitope (see Fig. 2 *b*). (*b*) Immunoblots reacted with the anti–42-kD mAbs of fractions isolated during an SPB preparation. Fraction numbers are the same as those in Rout and Kilmartin (1990). SPBs are mainly in fraction 4 of the sucrose gradient and fraction 2 of the Percoll gradient. (*c*) Immuno-EM of isolated SPBs using the anti–42-kD mAbs. Bars: (*a*) 2.5 μm; (*c*) 0.1 μm.

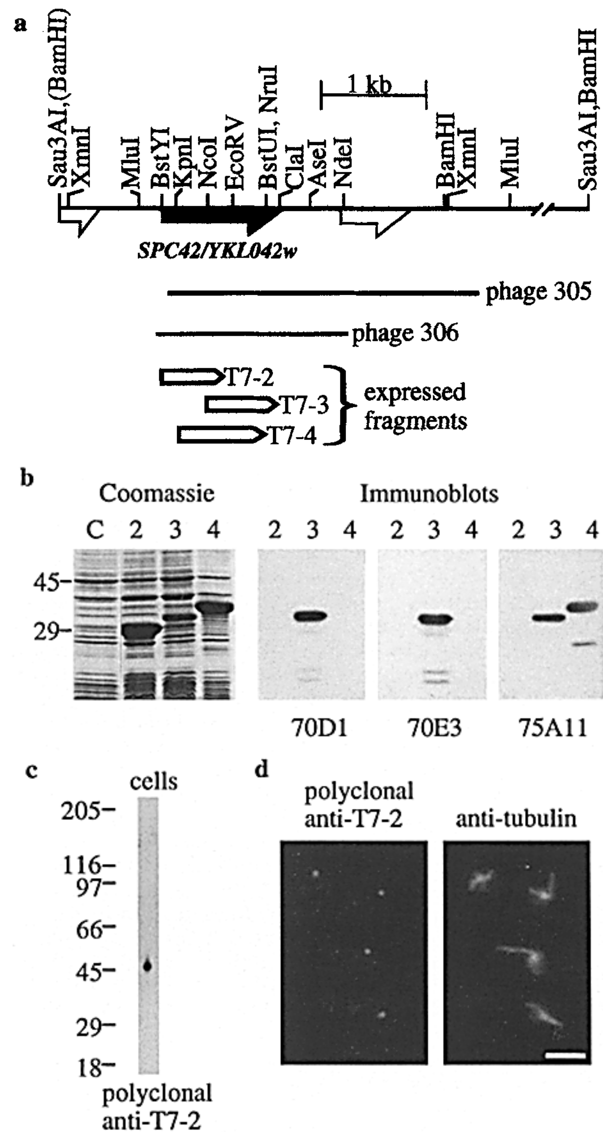


Figure 2. (*a*) Restriction map of *SPC42*, diagram of the inserts of λgt11 phage isolated by screening a yeast genomic DNA expression bank with the anti–42-kD mAbs, and diagram of fragments expressed in *E. coli*. Other sites for Sau3AI, BstYI, NcoI, EcoRV, BstUI, and ClaI are present between the two XmnI sites shown. (*b*) Coomassie stained SDS gradient gel of the three Spc42p fragments expressed in *E. coli* together with the corresponding immunoblots reacted with the individual anti–42-kD mAbs. (*c*) Immunoblot of *S. uvarum* cell extract separated by a gradient SDS gel and reacted with a mouse polyclonal affinity-purified antibody prepared against fragment T7-2. (*d*) Immunofluorescent staining of *S. uvarum* cells with the mouse polyclonal affinity-purified antibody prepared against fragment T7-2. Bar, 2.5 μm.

tionated on Percoll and was again coincident with the SPB-containing fraction on this gradient (Fig. 1 *b*). The apparent molecular mass of ~48 kD was somewhat higher than previously measured (Rout and Kilmartin, 1991). The immunogold staining previously observed with the mAbs in enriched SPB preparations (Rout and Kilmartin, 1991) showed gold particles apparently bound to only one side of the central plaque. This suggested that the antigen was part of that structure. However this staining pattern is very similar to that obtained with antibodies against Cdc31p (Spang et al., 1993), a component of the bridge or half-bridge that is attached to one side of the central plaque (Byers, 1981*a*). This half-bridge structure is not very visible in the enriched SPB preparations from *Saccharomyces uvarum*. We reexamined the sections to distinguish between localization to the bridge, where only one side of the central plaque would be expected to stain, and localization to the central plaque itself where, depending on the penetration of the antibodies, a significant proportion of the SPBs should be stained on both sides of the central plaque (Fig. 1 *c*). We found that of the SPBs with gold particles attached to the central plaque, 28% (n = 129) had particles attached to both left and right sides, strongly suggesting that the 42-kD component is part of the central plaque.

Cloning of SPC42

The three anti–42-kD mAbs were pooled and used to screen a λgt11 yeast genomic DNA expression bank. Two classes of positive phage were isolated (Fig. 2 *a*). Prelimi-

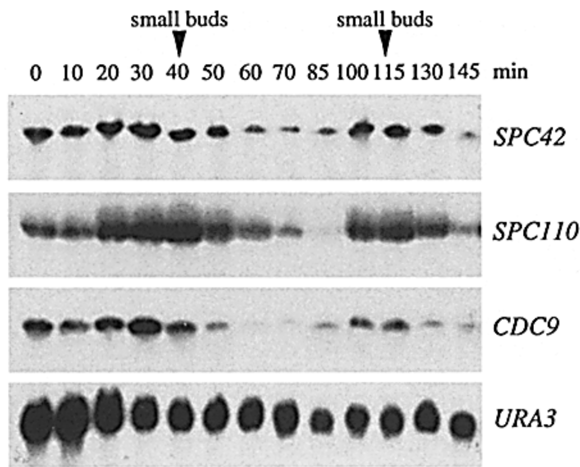


Figure 3. Northern blots of total RNA isolated from cells synchronized with α-factor using probes against *SPC42*, *SPC110*, *CDC9*, and with *URA3* as a loading control. Arrowheads indicate when small buds appear.

nary sequence data indicated an open reading frame of about the right size at the 5′ end of phage 306. To establish that this was indeed the correct gene we sought to confirm that the encoded protein shared several epitopes with the SPB component, since the epitope for a single mAb can often be present on unrelated proteins (Wehland et al., 1984). Three fragments of the open reading frame were expressed (Fig. 2 *a*) and their reactivity with the mAbs assessed from immunoblots (Fig. 2 *b*). None of the mAbs apparently reacted with fragment T7-2. Both 70D1 and 70E3 reacted only with fragment T7-3, while 75A11 reacted with both fragments T7-3 and T7-4. These results suggest two independent epitopes for the three mAbs. A further check of the gene's identity was made by preparation of an affinity-purified antibody against fragment T7-2, which apparently contains no mAb epitopes. This polyclonal antibody, unlike the mAbs, was able to detect the 48-kD protein in immunoblots of whole yeast cells (Fig. 2 *c*), and stained the SPB region in immunofluorescence of yeast cells (Fig. 2 *d*). These results define at least three epitopes of the spindle pole antigen also present in the cloned open reading frame, and strongly suggest that the correct gene had been cloned. We have named this gene *SPC42* (spindle pole component).

Some Features of the SPC42 Sequence

A larger fragment containing *SPC42* was isolated and the region containing the gene was sequenced. The open reading frame predicts a polypeptide of 363 amino acids and molecular mass 42.2 kD. While this work was in progress part of the sequence of chromosome 11 was published (Purnelle et al., 1993). It contains an open reading frame, YKL255, identical to Spc42p (YKL255 was subsequently called YKL042w in Dujon et al. [1994]). There are two interesting features in this sequence. First, in the predicted polypeptide there is a short region of potential coiled-coil structure between residues 55 and 135 (see Fig. 4 *b*). When the sequence was subjected to the BLAST homology search program (Altschul et al., 1990) the only matches found were with the coiled-coil regions of other proteins.

The second interesting feature of the sequence is the presence of a partial match to an MluI cell cycle box (MCB, consensus sequence ACGCGTNA) at positions −227 to −232 (ACGCGTCG) in the potential promoter region of *SPC42*. This position is within the range of −100 to −250 where MCBs are present in other genes (Johnston et al., 1991; McIntosh et al., 1991), including a potential MCB in *SPC110* (Kilmartin et al., 1993). We tested whether the *SPC42* mRNA was cell cycle regulated by probing a Northern blot of cells synchronized with α-factor. We detected a transcript of ~1.3 kb (data not shown), consistent with the size of the *SPC42* proposed open reading frame (1089 bp). Levels of this transcript showed some cell cycle regulation (Fig. 3). Its phase was similar to that of another MCB-regulated gene *CDC9* (White et al., 1986) and also *SPC110*. The extent of cell cycle regulation was somewhat greater than with another gene *DBF4* (Chapman and Johnston, 1989; Kitada et al., 1992) containing a similar partial match (ACGCGTCT) to the MCB.

ts Mutants in SPC42

An almost complete disruption of *SPC42* (see Materials and Methods) which removed all but 39 residues at the COOH terminus showed that the gene is essential. Thus to investigate the role of Spc42p in SPB function, we prepared ts mutants by PCR mutagenesis. Seven *spc42* alleles were obtained. Only two identical changes were found among the total of 42 amino acid alterations in the seven alleles (see Materials and Methods and Fig. 4 *a*.). However we noticed that in six of the seven alleles a change to a proline occurred in the potential coiled-coil region (Fig. 4, *a* and *b*). Such a mutation might disrupt the α-helix and cause the ts phenotype. We found that for all seven alleles transfer of a restriction fragment (see Materials and Methods) containing the changes in the region encoding the potential coiled-coil region to *SPC42* was sufficient to cause the ts phenotype. The single mutations of L121:P, L86:P, and Q110:P found in three of the alleles were constructed individually and all caused temperature-sensitive growth (Fig. 4 *c* and data not shown).

Phenotype of ts Mutants in SPC42

The phenotype of *spc42-10* cells was initially examined by immunofluorescence (Fig. 5). Cells were grown at 36°C for 4 h then fixed and stained with mAbs (Rout and Kilmartin, 1990) against the 90-kD SPB component (Fig. 5 *a*), anti-tubulin (Fig. 5 *b*) and DAPI to stain DNA (Fig. 5 *c*). Cells appeared to arrest with a single microtubule aster, similar to the monopolar spindle or *mps* phenotype (Winey et al., 1991*b*). About 85% of the cells observed had the *mps* phenotype, while 10% appeared to be aploid cells containing little DNA and 5% appeared to have two SPBs joined by an apparently normal short spindle. Longer post-anaphase spindles were not found. All the other alleles examined had a similar phenotype, suggesting a defect in SPB function. To look at the phenotype in more detail, *spc42-10* cells were examined by EM of serial thin sections. Cells at the permissive temperature showed normal SPBs (Fig. 6 *a*). However cells arrested at 36°C for 4 h

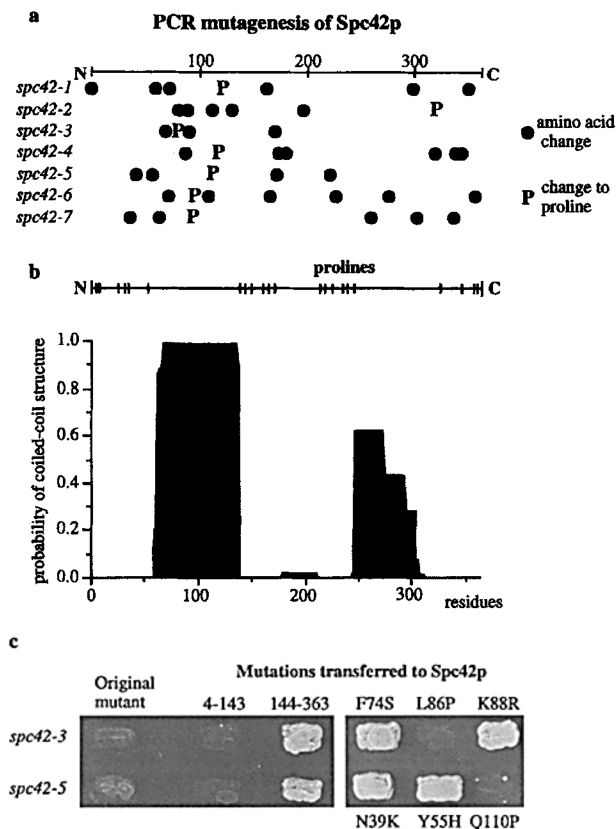


Figure 4. (*a*) Diagram of the amino acid changes induced by PCR mutagenesis of *SPC42* for each of the *spc42* alleles. Filled circles show an amino acid change and P shows a change to a proline. (*b*) Distribution of proline residues and probability of coiled-coil structure in Spc42p (Lupas et al., 1991). (*c*) Growth of *spc42* alleles at 36°C. In the left panel restriction fragment exchange (see Materials and Methods) was used to separate the mutations in the NH_2-terminal (amino acids 4–143) and COOH-terminal (144–363) portions. In the right hand panel the three individual mutations contained between 4–143 were constructed singly by site-directed mutagenesis.

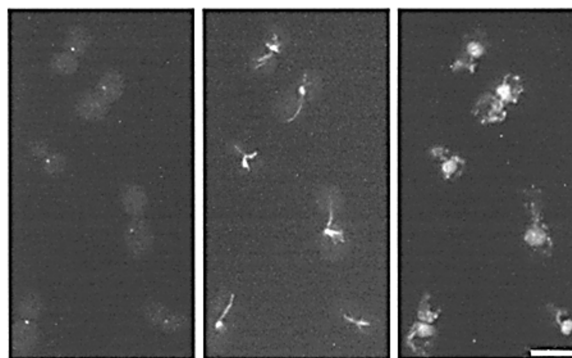
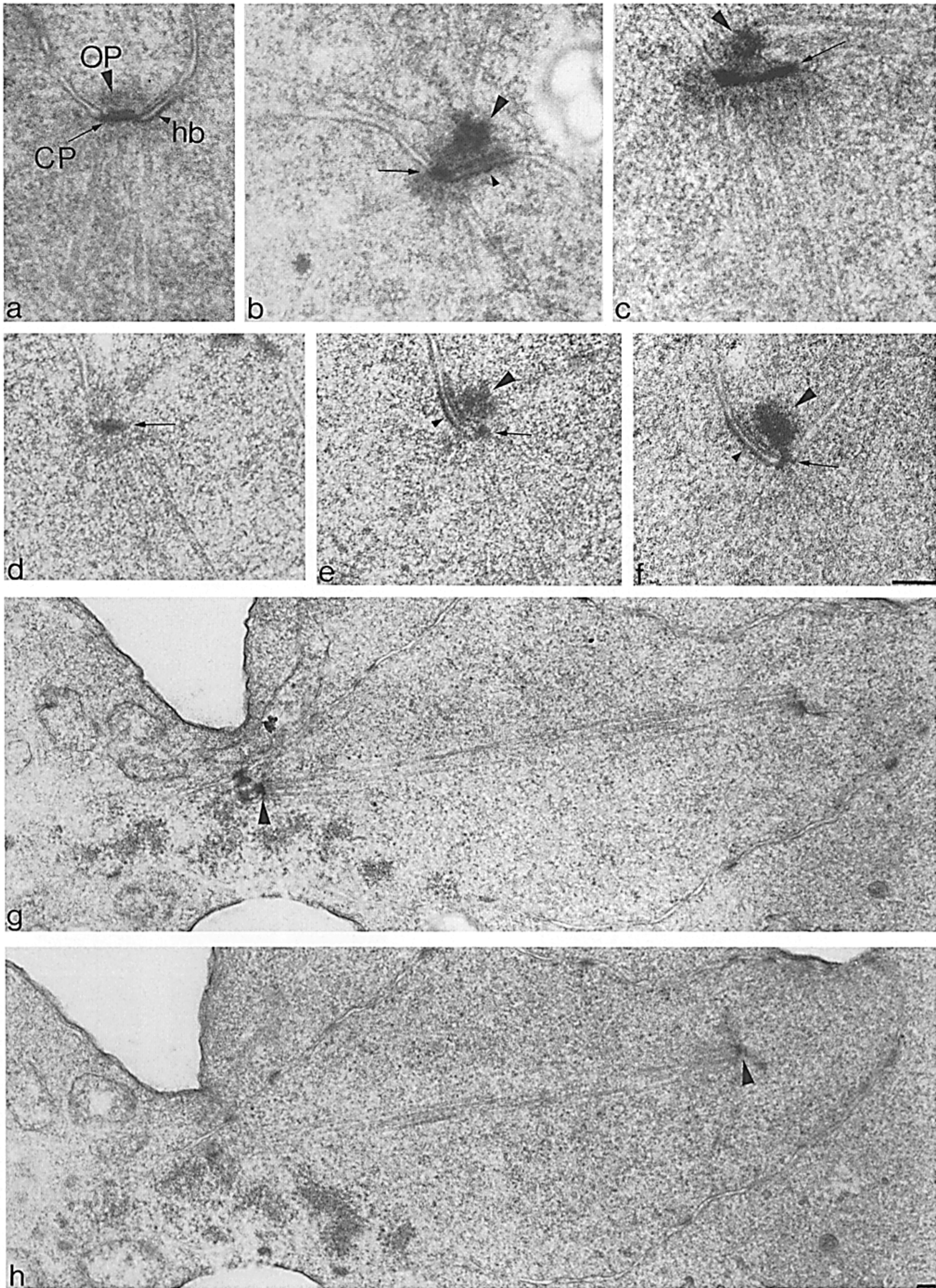


Figure 5. Immunofluorescent staining of *spc42-10* cells grown at 36°C for 4 h. Cells were stained for SPBs with a mix of the anti–90-kD mAbs (*left*), rabbit anti-yeast tubulin (*middle*) and DAPI for DNA (*right*). *spc42-10* contains the single mutation Q110:P. Bar, 5 μm.

showed a variety of abnormal morphologies for all of the SPBs examined. The central plaque had a smaller diameter (Fig. 6 *b*) and from analysis of serial sections often had a noncircular shape (Fig. 6, *d–f*). The outer plaque was often indistinct, the half-bridge was large and there was usually an electron-dense mass above the bridge (Fig. 6, *b–f*). Occasionally cytoplasmic microtubules seemed to be associated with this electron-dense mass (Fig. 6, *b* and *c*). Cross-sections of nuclear microtubules close to the SPBs showed 17–23 microtubules, which is very similar to the 19–24 microtubules found in wild-type haploid SPBs (Peterson and Ris, 1976), suggesting no defect in microtubule initiation. Some SPBs had a bilobed appearance (Fig. 6 *c*) and others seemed to have completed a duplication or fission process to form a "spindle" (Fig. 6, *g* and *h*). It was often not clear whether one end of these spindles was properly attached to the nuclear envelope, though attachment via a thin involution of the envelope would be difficult to detect. SPBs were examined in 45 cells, in which 18 had apparently single SPBs, 17 had bilobed SPBs, and 10 had spindles. A more systematic examination by complete serial sectioning of the nuclei of 16 randomly selected cells showed 10 with single SPBs, 2 with bilobed SPBs, 3 with spindles (one was tripolar), and 1 with two independent SPBs. EM of *spc42-11* cells (see Materials and Methods) showed a similar range of phenotypes. These results suggest a defect in normal SPB duplication since most cells have a single or bilobed SPB. The accumulation of electron-dense material associated with the cytoplasmic surface of the bridge is also indicative of a failure in the duplication process since this is where the new daughter SPB would assemble in wild-type cells (Byers and Goetsch, 1974; Winey et al., 1991*a*).

We decided to examine SPB duplication in detail by using cells synchronized in G_1 by elutriation. Such cells are selected on the basis of their very small size and require ~45 min of growth before passing through Start and beginning DNA replication (Lew et al., 1992). We classified the SPBs of such cells by EM, and also measured the budding index and the stage of DNA replication by flow cytometry (Fig. 7). Wild-type cells start to bud at 1 h at 36°C, pass through both SPB and DNA replication between 1 and 1.5 h, and then through mitosis between 1.5 and 2 h. In comparison *spc42-10* cells passed through both budding and DNA replication with only a slight delay but only completed one round of each process. These cells did not duplicate their SPBs at the appropriate time, but instead the budded cells remained with single SPBs. At later time points electron-dense material similar to that seen in Fig. 6, *b–f* accumulated in association with these SPBs. At very late time points of 5.5 h some cells contained bilobed SPBs and abnormal spindles similar to those in Fig. 6, *g* and *h*. These results can explain some of the heterogeneity ob-

Figure 6. EM of thin sections of *spc42-10* cells grown at 23°C (*a*) or for 4 h at 36°C (*b–h*). Some features of the SPB are shown in *a*, the central plaque (*CP*), the outer plaque (*OP*), and the half-bridge (*hb*). In *b–f* the central plaque is shown by an arrow, the half-bridge by a small arrowhead, and the cytoplasmic electron-dense mass by a large arrowhead. In *g* and *h* the arrowhead shows the spindle poles. *d–f* and *g–h* are serial sections. Bar, 0.1 μm.


OP
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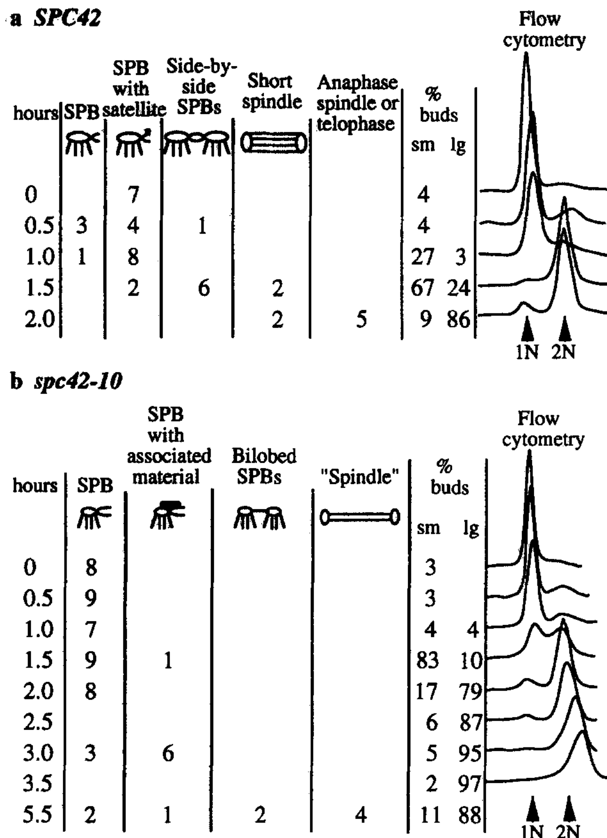

a *SPC42*

hours	SPB	SPB with satellite	Side-by-side SPBs	Short spindle	Anaphase spindle or telophase	% buds sm	% buds lg
0		7				4	
0.5	3	4	1			4	
1.0	1	8				27	3
1.5		2	6	2		67	24
2.0				2	5	9	86

b *spc42-10*

hours	SPB	SPB with associated material	Bilobed SPBs	"Spindle"	% buds sm	% buds lg
0	8				3	
0.5	9				3	
1.0	7				4	4
1.5	9	1			83	10
2.0	8				17	79
2.5					6	87
3.0	3	6			5	95
3.5					2	97
5.5	2	1	2	4	11	88

Figure 7. Classification of SPBs by EM, budding index and flow cytometry in cells synchronized by elutriation and released at 36°C. (*a*) *SPC42* and (*b*) *spc42-10*. For the *spc42-10* cells the SPBs with associated material correspond to those illustrated in Fig. 6, *b* to *f*, the bilobed SPBs to that in Fig. 6 *c*, and the spindle to that in Fig. 6, *g* and *h*. The budding index is divided into small (*sm*), where buds are less than approximately one third of the mother and large (*lg*), all larger buds. *SPC42* and *spc42-10* are isogenic apart from the Q110:P mutation.

served in the phenotype of asynchronous cells after 4 h at 36°C. Cells shifted to 36°C early in their cell cycle have the time to produce bilobed SPBs and abnormal spindles whereas those that undergo the temperature shift later in the cell cycle later show the single SPB phenotype.

In addition to the EM, budding index and flow cytometry measurements, we also measured the viability of these elutriated cells during the course of the cell cycle after release at 36°C. Such studies can be valuable in establishing when in the cell cycle an essential gene product is required, since there is a often a sharp decrease in viability after passing through that particular point in the cell cycle (Davis, 1992; Holm et al., 1985; McGrew et al., 1992). At 36°C *spc42-10* cells synchronized by elutriation show a sharp drop in viability at the time of normal SPB duplication (Fig. 8 *a*). This result, together with the EM evidence showing abnormalities in SPB duplication, suggested that an irreversible defect in the process of SPB duplication itself was causing the lethality. To confirm this we inhibited SPB duplication in the elutriated *spc42-10* cells by incubation with α-factor at 36°C (Byers and Goetsch, 1974). Such cells remained viable for up to 6 h (Fig. 8 *a*). If the α-factor was removed from such cells after 3 h, thereby allowing

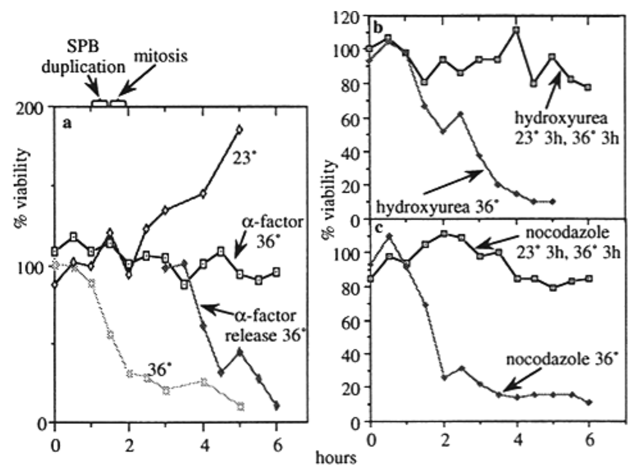

Figure 8. Viability of *spc42-10* cells synchronized by elutriation. (*a*) Cells released at 23°C (*dotted line with open diamonds*) or 36°C (*dotted line with squares*), cells released at 36°C in α-factor (*full line with open squares*) or the same cells at 36°C with α-factor removed at 3 h (*dotted line with solid diamonds*). (*b*) Cells released at 36°C in hydroxyurea (*dotted line with diamonds*) or released for 3 h at 23°C then 3 h at 36°C in hydroxyurea (*full line with squares*). (*c*) Cells released at 36°C in nocodazole (*dotted line with diamonds*) or released for 3 h at 23°C then 3 h at 36°C in nocodazole (*full line with squares*). Incubation of control cells, *spc42-10* at 23°C or *SPC42* at 36°C, showed no significant viability decreases under the above conditions.

them to attempt SPB duplication, viability decreased sharply (Fig. 8 *a*). Further confirmation that the lethality was associated with SPB duplication came from treatment of elutriated *spc42-10* cells with either hydroxyurea (Fig. 8 *b*) or nocodazole (Fig. 8 *c*) at 36°C. Hydroxyurea inhibits DNA synthesis while nocodazole inhibits mitosis, but both these

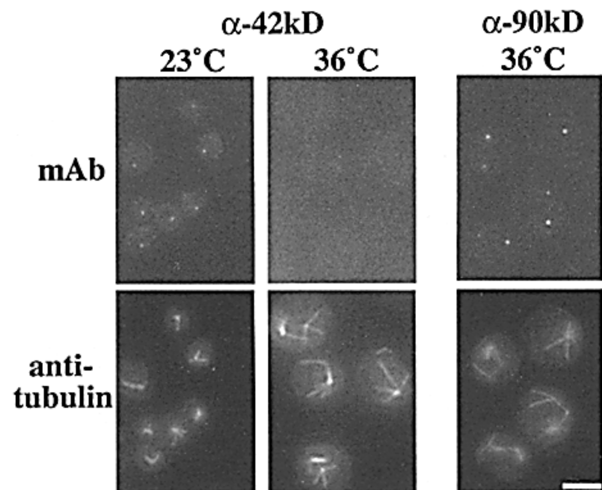

Figure 9. Immunofluorescence of *spc42-10* cells grown at 23°C (*left*) or for 4 h at 36°C (*middle and right*) then rapidly spheroplasted at 23 or 36°C, respectively, fixed in methanol and acetone and stained with anti–42-kD or anti–90-kD mAbs (*top*) and anti-tubulin (*bottom*). The variable intensities of anti–90-kD staining within single cells probably reflect fission or break up of the SPBs as was found by EM (Fig. 6, *g* and *h*). The weak spots of anti–90-kD staining always coincide with the junctions between microtubules. Bar, 4 μm.

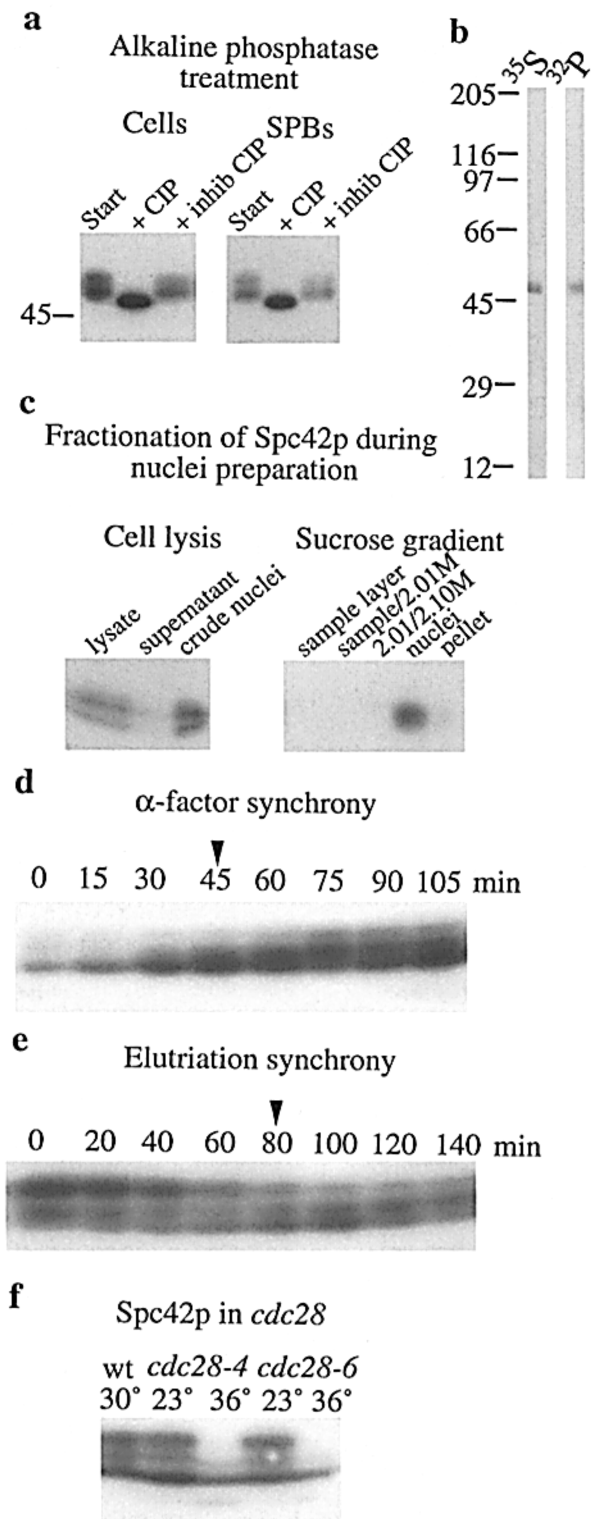

Figure 10. (*a*) Calf intestinal alkaline phosphatase (*CIP*) treatment of Spc42p immunoprecipitated from *S. cerevisiae* (K699) cells (*left*) or enriched SPBs (without immunoprecipitation) from the same strain (*right*). Samples were separated with SDS gradient gels, immunoblotted then reacted with polyclonal affinity-purified anti-Spc42p. As a control 0.1 M β-glycerophosphate was added to inhibit the alkaline phosphatase (*inhib CIP*). (*b*) Autoradiographs of SDS gradient gels of immunoprecipitated Spc42p from K699 cells labeled with S^{35} (*left*) or P^{32} (*right*). (*c*) Immunoblots of fractions isolated during nuclei preparation from *S. uvarum* were reacted with polyclonal affinity–purified anti-Spc42p. (*d* and *e*) Immunoblots of K699 cells separated by SDS gradient gels and reacted with polyclonal affinity-purified anti-Spc42p. Cells were synchronized by α-factor (*d*) or elutriation (*e*) and released at 30°C, gel tracks contained equal protein loadings. Arrowheads indicate when small buds appear. (*f*) Immunoblot of K699 cells (*left*), *cdc28-4* and *cdc28-6* cells grown at 23 or 36°C separated by a gradient SDS gel and reacted with polyclonal affinity-purified anti-Spc42p. Note that these two alleles actually have the same sequence change (Lorincz and Reed, 1986), the two sets of results are included to show that the different genetic backgrounds made no difference.

reagents allow SPB duplication (Byers and Goetsch, 1974 Hartwell, 1976; Jacobs et al., 1988) and neither arrested the decline in viability of *spc42-10* cells (Fig. 8, *b* and *c*). If however the elutriated *spc42-10* cells were allowed to duplicate their SPBs normally at 23°C in either hydroxyurea or nocodazole then incubated at 36°C viability remained normal (Fig. 8, *b* and *c*).

The EM results suggest that in *spc42-10* cells at 36°C a structural alteration occurs in the central plaque of all of the SPBs. One possible cause of this alteration might be that the thermolabile Spc42p becomes dissociated from the SPB at 36°C. We tested this by staining *spc42-10* cells after incubation for 4 h at 36°C with both anti-Spc42p and anti–90-kD antibodies. The 90-kD staining was still present but the staining intensity with the anti–42-kD antibodies was greatly reduced (Fig. 9).

Spc42p Is a Phosphoprotein

The availability of monospecific antibodies capable of detecting Spc42p in immunoblots of cell extracts (Fig. 2 *c*) allowed a further characterization of the protein. We used large format shallow (8–13%) SDS gradient gels that gave greater resolution than the minigels used previously (Fig. 2 *c*). We now found that immunoblots from these longer gels showed that the protein migrated as a heterogeneous group of bands of apparent molecular mass between 46 and 51 kD (Fig. 10 *a*). Such behavior is often a consequence of hyperphosphorylation (Papkoff et al., 1982). This was confirmed by digestion of immunoprecipitated Spc42p with alkaline phosphatase, which resulted in an apparently homogeneous band of ~44 kD (Fig. 10 *a*), slightly higher than the expected size of 42 kD for Spc42p. Inhibition of alkaline phosphatase by β-glycerophosphate left the heterogeneous pattern largely unchanged (Fig. 10 *a*). Similar results were obtained from SPBs isolated from the same strain (Fig. 10 *a*). A more direct demonstration that Spc42p is a phosphoprotein was by immunoprecipitation from ^{32}P-labeled cells. Control experiments using ^{35}S-labeled cells showed that immunoprecipitates consisted almost exclusively of heterogeneous bands with a similar mobility to those seen above (Fig. 10 *b*). In ^{32}P-labeled cells, these heterogeneous bands were labeled (Fig. 10 *b*), showing directly that Spc42p is a phosphoprotein.

The mobility and distribution of the heterogeneous Spc42p bands in whole cells and in isolated SPBs from the same strain is quite similar suggesting that there is no pool of unassembled Spc42p in a different phosphorylation state. This was partly confirmed from analysis of fractions

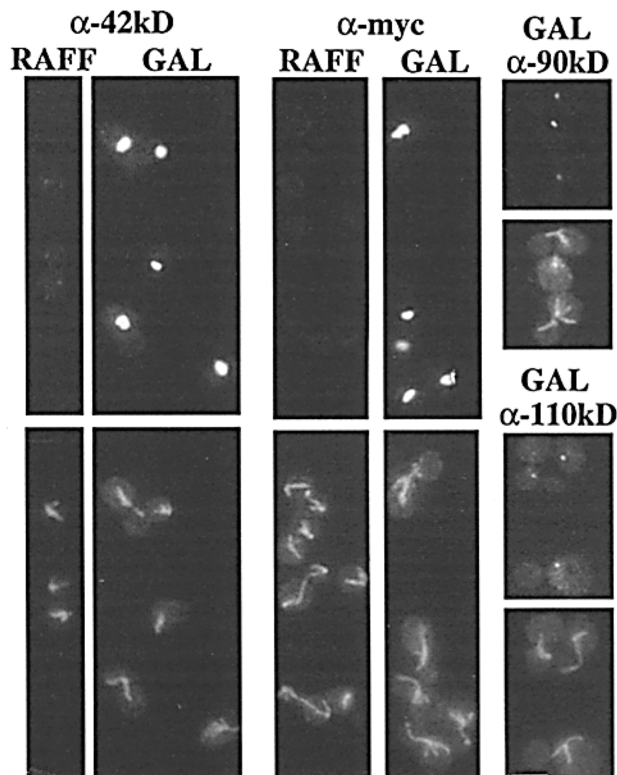

Figure 11. Immunofluorescence of JK813c cells in raffinose (*RAFF*) or after induction of myc-tagged Spc42p in galactose (*GAL*) for 3 h at 30°C. Cells were stained with a pool of anti–42-kD mAbs, or anti-myc mAb 9E10, or pools of mAbs against either the 90-kD or the 110-kD SPB components. Anti-tubulin staining is shown in the bottom panel of each pair. Bar, 3.5 μm.

taken during the nuclei preparation. Practically all the Spc42p cofractionates with the nuclei (Fig. 10 *c*). When the nuclei are fractionated all the Spc42p cofractionates with the SPBs (Fig. 1 *a*). However since these SPB preparations are only 10% pure (Rout and Kilmartin, 1990), there might be an insoluble fraction of Spc42p that is not SPB-associated but happens to cofractionate.

Is the Phosphorylation of Spc42p Cell Cycle Regulated?

The Cdc28p/cdc2 kinase controls the initiation of Start (Hartwell, 1974) and an early event after Start is SPB duplication. Since Spc42p has an essential role at the time of SPB duplication and is a phosphoprotein, it could potentially be a substrate for the Cdc28p kinase or possibly other cell cycle kinases such as Mps1p (Lauze et al., 1995) or Bub1p (Roberts et al., 1994). To test this we first determined whether the phosphorylation of Spc42p was cell cycle regulated. Cells synchronized by α-factor did show increases in phosphorylation during the cell cycle particularly in the case of the middle band (Fig. 10 *d*). Elutriated cells behaved similarly after budding (after arrowhead in Fig. 10 *e*); however before budding Spc42p appears to be hyperphosphorylated in these cells. This may reflect the fact that elutriated cells are very small and thus at an earlier stage of G_1 than α-factor–blocked cells which are full sized. *cdc28* cells also arrest as full-sized G_1 cells, and these gave a similar gel band pattern to α-factor–blocked cells suggesting that Spc42p is dephosphorylated (Fig. 10 *f*). Thus there are clear differences in the band pattern between G_1 cells and cells at other stages of the cell cycle. There also appear to be differences in the apparent amounts of Spc42p at different cell cycle stages.

One possible problem with the interpretation of the band patterns of the synchronized cells after budding is that during the time that Spc42p is being phosphorylated extra protein is probably being made due to the increased transcript level (Fig. 3). So the apparent cell cycle-regulated phosphorylation could be merely constitutive phosphorylation of this newly synthesized protein. To distinguish between these two possibilities we examined the endogenous Spc42p in a series of *cdc* mutants that define various points in the cell cycle (Din et al., 1990; Foiani et al., 1995). There was a gradual decrease in mobility of Spc42p in mutants with later blocks in the cell cycle, but the band patterns were rather complex (data not shown). Since the relationship between band shift and phosphorylation is dependent on the particular residue being phosphorylated (Gustke et al., 1992), a better way to analyze the potentially different phosphorylation states of Spc42p would be by peptide mapping (Moll et al., 1991). In that way the phosphorylation of individual residues could be correlated with the position of the cells in the cell cycle.

Overexpression of SPC42

The potential coiled-coil region of Spc42p suggests that the protein has a structural role in the central plaque. Because such proteins are often capable of self-assembly we investigated whether overexpression in yeast would lead to the formation of a polymer that would provide some insights as to the function of Spc42p in the central plaque. We used the inducible GAL promoter and myc-tagged Spc42p to distinguish it from the endogenous Spc42p. The myc-tagged protein is functional since under the control of the wild-type promoter it complements a deletion of *SPC42* (see Materials and Methods). Prolonged overexpression of *SPC42* was toxic since the yeast strain containing this construct did not grow on galactose plates; however viability was reasonable (~50%) after a shorter period of induction of 3 h. Immunofluorescence showed a large increase in the area of staining with both anti–42-kD and anti-myc mAbs coincident with the microtubule aster (Fig. 11). The size of the stained area was dependent on the length of induction but always centered on the microtubule aster (data not shown). The extent of staining with both anti–110-kD and anti–90-kD mAbs remained the same as in wild-type cells (Fig. 11). There were very few mitotic cells present. We examined these cells by EM to see if any novel structure was detectable. A large dome-shaped electron dense structure was present which appeared to be attached to the central plaque and was surrounded on one side with the cytoplasmic surface of the nuclear membrane and on the other by what appeared to be ER (Fig. 12 *a*). The thickness of this novel structure was fairly uniform and similar to the central plaque. There appeared to be some regularity in the structure since at certain sectioning angles striations were apparent (Fig. 12 *b*), and an en face view showed a lace-like packing with some regularity in the spacing (Fig. 12 *c*).

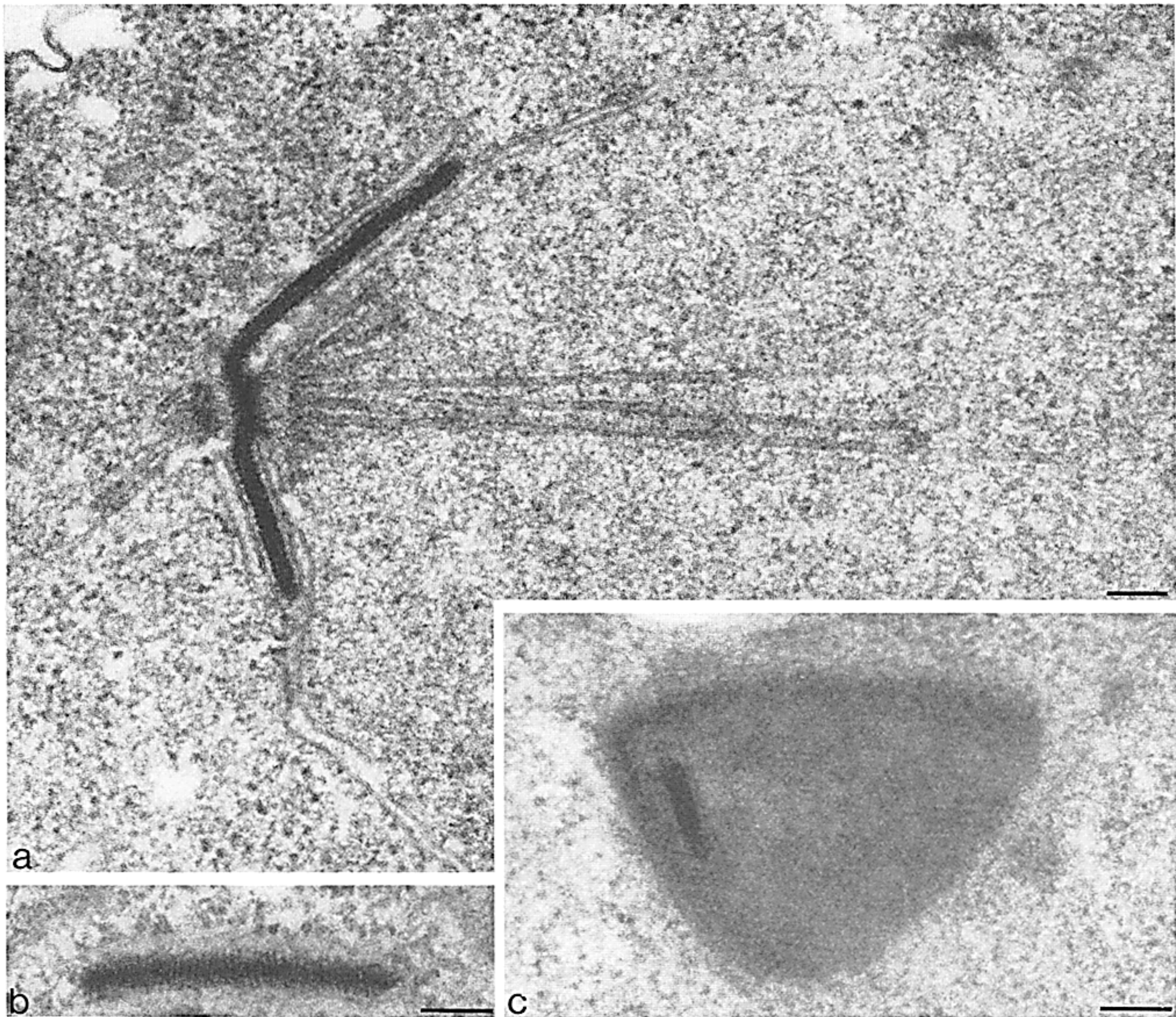

Figure 12. (*a*) EM of a thin section of a JK813c cell incubated in galactose medium for 3 h at 30°C to induce synthesis of myc-tagged Spc42p. (*b*) Cross-section of the Spc42p polymer to show vertical striations and (*c*) en face view to show a lace-like array of filaments. Bar, 0.1 μm.

To check whether Spc42p was associated with other proteins in this structure we attempted to purify it using our SPB enrichment procedure followed by heparin extraction (Rout and Kilmartin, 1990). Enriched SPBs still retained the structure which was clearly attached firmly to the central plaque (Fig. 13 *a*). SDS gels showed extra heterogeneous bands between 55 and 51 kD on comparison with SPBs from uninduced cells (Fig. 13 *b*). Heparin extraction of the enriched SPBs further purified the structures so that a pellet viewed from top to bottom was quite homogeneous (Fig. 13 *c*). SDS gels showed the same-sized heterogeneous bands, while SPBs from uninduced cells showed no detectable bands from the same gradient fraction (Fig. 13 *d*). The heterogeneity was due to hyperphosphorylation as in the wild-type Spc42p since digestion with alkaline phosphatase produced a homogeneous band of lower molecular mass of 49 kD, somewhat higher than the expected size of 43.6 kD for myc-tagged Spc42p. All of these bands reacted with both the anti–42-kD mAbs and the anti-myc mAb 9E10 (Fig. 13 *e*). These results show that Spc42p is capable of forming a filamentous polymer, apparently initiated from the central plaque, which interacts with the outer nuclear membrane and probably ER and is composed of phosphorylated Spc42p.

Discussion

Common Features of Two SPB Plaque Components

SPC42 shares two features with the gene encoding another plaque component *SPC110* (Rout and Kilmartin, 1990; Kilmartin et al., 1993). First, both have MCBs that may act to produce new transcripts at the G_1/S transition when the new SPB is assembled (Byers, 1981*a*). Other plaque components may also be under the same control. In contrast, two other gene products localized to the half-bridge of the

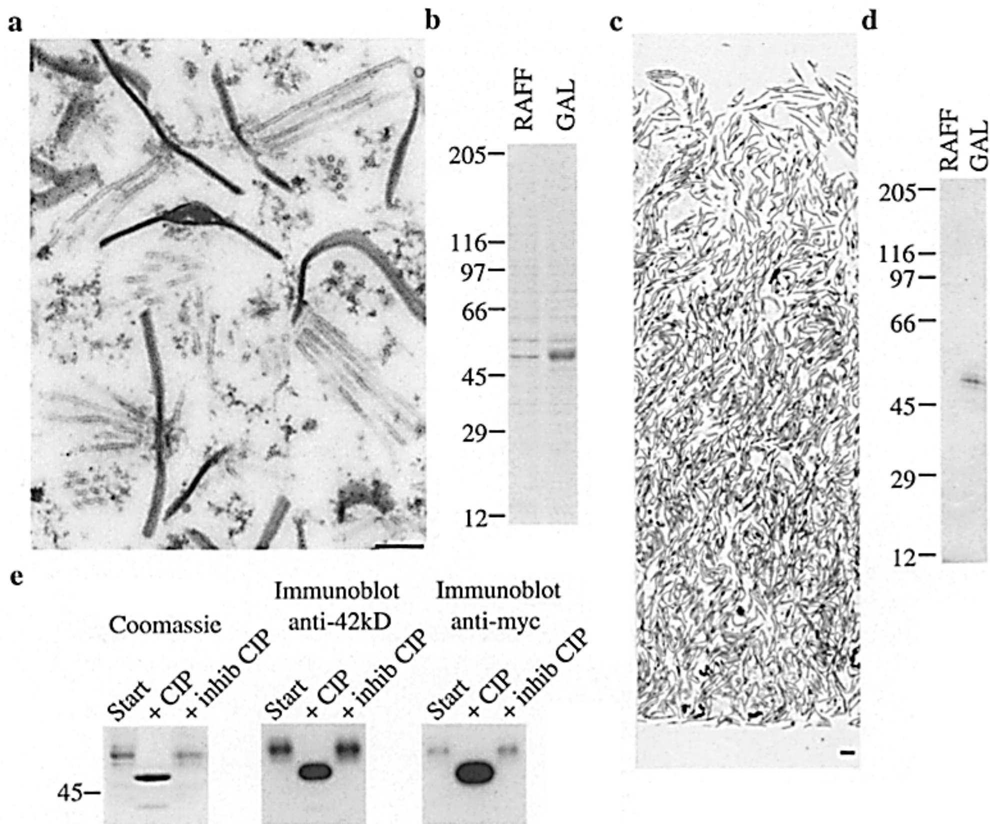

Figure 13. (*a*) EM of a thin section of part of a pellet of SPBs isolated from JKY813c cells induced with galactose and (*b*) an SDS gradient gel of the same fraction from uninduced cells (*RAFF*) and cells induced as above (*GAL*). (*c*) EM of a thin section from the top to the bottom of a pellet of heparin-treated SPBs isolated from the same induced strain and (*d*) an SDS gradient gel of the same fraction from uninduced cells (*RAFF*) and cells induced as above (*GAL*). (*e*) The left panel shows in order a Coomassie stained gel of the heparin-treated SPBs from *d* (*Start*), the same material treated with alkaline phosphatase (*CIP*) and as a control the alkaline phosphatase was inhibited with 0.1 M β-glycerophosphate (*inhib CIP*). The middle and right hand panels show the same gel immunoblotted and reacted with anti–42-kD antibodies or 9E10 anti-myc antibodies, respectively. Bar, 0.5 μm.

SPB, Cdc31p and Kar1p (Spang et al., 1993, 1995), do not have MCBs in their upstream sequences. Indeed Cdc31p has been shown to execute its essential function before SPB duplication (Winey et al., 1991*a*).

Second, both Spc42p and Spc110p contain regions of coiled-coil homology. The central coiled-coil rod of Spc110p acts as a spacer element between the central plaque and the nuclear microtubules (Kilmartin et al., 1993). In the case of Spc42p we cannot be sure that the region between residues 55 and 135 forms a coiled-coil, since the predicted length is too short to observe by EM, however the Lupas (Lupas et al., 1991) probability score for coiled-coil structure is high (0.99). The substitution of the helix-perturbing residue proline into this region to give the ts phenotype also suggests an α-helical conformation, and further suggests that this coiled-coil region is essential for Spc42p function.

Possession of both a potential MCB box and a coiled-coil region is not exclusive to SPB plaque components. Members of the SMC family of proteins *SMC1* and *SMC2* (Strunnikov et al., 1993, 1995) also have both these features and they, together with other members of the family (Hirano and Mitchison, 1994), clearly function in chromosome condensation and not at the spindle poles.

Phenotype of spc42

Initial analysis of the phenotypes of the *spc42* alleles by EM showed a complex spectrum of abnormal morphologies, including cytoplasmic electron-dense masses close to the half-bridge, changes in central plaque shape, and spindles, some of which have one or both poles apparently detached from the nuclear membrane. Closer study showed that these pleiotrophic phenotypes appear to arise after the failure of SPB duplication and as Spc42p is lost from SPBs.

The cytoplasmic electron-dense masses appear late in the synchronized cell phenotype. Their association with the half-bridge suggests that they represent material accumulated after continued failure of SPB assembly due to lack of Spc42p function. In addition to the defect in SPB duplication, mutation of Spc42p appears to affect existing SPBs, since we observe abnormal SPBs in the mutant cells after several hours at 36°C. The changes in central plaque shape may be due to the loss of Spc42p from the SPB, as shown by the reduction in immunofluorescence. The formation of spindles at late stages in the block could be a result of the action of mitotic kinesin-like proteins such as Kip1p and Cin8p (Saunders and Hoyt, 1992). In attempting spindle formation these motors might be capable of wrenching a bilobed SPB into two pieces and possibly detaching them from the nuclear membrane.

Why does the failure of SPB duplication cause a decrease in the viability of *spc42-10*? It appears that potential SPB duplication checkpoint systems (Weiss and Winey, 1996) do not detect the *spc42-10* duplication failure, though the cell cycle is arrested. One possibility could be that the SPB can only be duplicated during a discrete interval during the cell cycle, so if cells are returned to permissive conditions too late in the cell cycle they may fail to recover. A second possibility could be that the effects of the mutation irreversibly affect the competence of the half-bridge to nucleate SPB duplication (see below).

The spindle pole phenotype of *spc42* is different from that of other known genes involved in SPB duplication, since ts mutants in both *CDC31* and *KAR1* (whose gene products localize to the half-bridge) and in *MPS1* (which encodes a protein kinase) all arrest with a single enlarged SPB. It appears that in these mutants the assembly of the plaque components is normal, but instead of initiating on the satellite and bridge to form a daughter SPB, they assemble around the preexisting mother SPB. These gene products therefore appear to be involved in defining the position of the initiation of SPB duplication and thus it seems likely that these three genes act earlier in the SPB assembly pathway than *SPC42*.

Mutations in plaque components other than Spc42p might lead to similar phenotypes due to irreversible defects in SPB duplication. However, it is also possible to imagine more subtle defects. For example SPB duplication might appear normal but one or both SPBs could be defective. Even if this initial defect was reversible, later on in the cell cycle irreversible changes might occur if the cells attempted to pass through mitosis with such defective SPBs. Ts mutants in a gene *SPC110* encoding another plaque component appear to be of this class (Goh, P.-Y. and J.V. Kilmartin, manuscript in preparation).

Possible Functions of Spc42p

Analysis of the overexpression data can provide some insights into the relationship of Spc42p with the nuclear envelope and the central plaque. Overexpression leads to the formation of an electron-dense disc or dome-shaped polymer. The rapid formation of this structure in all the cells of an asynchronous population suggests a dynamic association of Spc42p with the SPB. This structure bears some resemblance to the central plaque and is apparently composed only of Spc42p. Could the central plaque itself be composed mainly of Spc42p? This seems unlikely since *spc42-10* cells still have a residual central plaque yet have undetectable Spc42p in it. Also a more detailed look at both the immuno-EM and overexpression data suggest a more complex structure for the central plaque. Most of the gold particles actually localize more towards the cytoplasmic side of the central plaque as seen in Rout and Kilmartin (1991) and Fig. 1 *c*. This could of course merely reflect accessibility; however the overexpression results also show that the Spc42p polymer is associated with the central plaque in exactly the same relative position (Fig. 12 *a* and lower two SPBs in Fig. 13 *a*). These results together suggest that Spc42p would occupy a peripheral cytoplasmic subsection of the central plaque. A more detailed structural analysis of the Spc42p polymer may indicate more precisely how this arrangement occurs.

The overexpression data shows a close relationship of the Spc42p polymer with the outer nuclear membrane, suggesting a role for Spc42p in attaching the SPB to the nuclear membrane. This possibility is supported by the mutant phenotype, where in about half the spindles (Fig. 6, *g* and *h*), either one or both SPBs were apparently detached from the nuclear membrane. Since Spc42p has no potential transmembrane domain, any membrane attachment must presumably be via another protein or a posttranslational modification.

A possible function for Spc42p, given its polymeric nature, its probable location to the periphery of the central plaque, and its possible role in nuclear membrane binding, might be a kind of molecular washer. A layer of Spc42p would surround other central plaque components possibly defining the size of the central plaque and facilitating insertion of the SPB into the nuclear membrane. This model for Spc42p could explain some aspects of the phenotype of *spc42*. Thus when mutant cells attempt SPB duplication we suggest that assembly of the daughter SPB on the half-bridge starts as normal. However in the absence of functional Spc42p that would normally surround the structure and prevent further growth, other SPB material continues to build up eventually forming the cytoplasmic electron-dense masses. This build up could irreversibly block access to the half-bridge so that SPB duplication is prevented even at the permissive temperature, thus mitosis fails and the cells die. The observed dissociation of the mutant protein from the central plaque of the existing SPBs could be responsible for the variation in shape. This model for Spc42p function also allows for the observed formation of SPBs of different diameters in haploid or diploid cells (Byers and Goetsch, 1974), since it is clear from the overexpression that Spc42p can form polymeric structures of varying diameter.

Phosphorylation of Spc42p

Spc42p is the first phosphoprotein identified in the SPB of *S. cerevisiae*, although there is good evidence that phosphoproteins are present in the SPB regions of other fungi. The mAb MPM-2, specific for mitotic phosphoproteins (Davis et al., 1983), stains the spindle poles of both *Aspergillus nidulans* (Engle et al., 1988) and *Schizosaccharomyces pombe* (Masuda et al., 1992). However, Spc42p does not appear to react with MPM-2 (data not shown).

What role might the phosphorylation of Spc42p play? One obvious model might be that it controls the assembly of Spc42p into the SPB. Our data on this model are contradictory if the phosphorylation is measured solely by gel shift. Spc42p migrates close to the dephosphorylated band position in late G_1 cells arrested by either α-factor treatment or the *cdc28* block. Both sets of cells have Spc42p in their SPBs (data not shown) which would suggest that the assembled form is dephosphorylated. But the rest of the data suggests an opposite conclusion. Thus when the arrested cells are allowed to reenter the cell cycle, or when cells are synchronized by elutriation, the mobility of Spc42p decreases during daughter SPB assembly, suggesting increased phosphorylation. Moreover, elutriated cells that are in early G_1 initially have a gel pattern suggesting Spc42p is hyperphosphorylated. Yet as these cells enter the cell cycle Spc42p is apparently dephosphorylated, returning to a gel mobility similar to that seen with α-factor-treated cells after budding. The complexity of the changes suggests that the role of phosphorylation may not be straightforward, and that there may be a number of phosphorylation sites having different functions. Use of the overexpressed Spc42p may allow direct identification of the different sites, which could then be confirmed and mutated in the endogenous protein. This would allow assay of the function of specific phosphorylation sites of the pro-

tein. The kinases involved might be identified by examining the phosphorylation state and assembly of Spc42p in various kinase mutants. Such experiments could provide insights into the pathway of SPB assembly and the specific role of Spc42p.

We thank D. Kershaw for cutting the serial thin sections, S. Dyos for preparing the overexpression strain, A. Knight for the Macintosh version of the Lupas program, and S. Holmes, S. Robinson, and H. Pelham for discussion.

Received for publication 3 October 1995 and in revised form 30 November 1995.

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