The 110-kD Spindle Pole Body Component of *Saccharomyces cerevisiae* Is a Phosphoprotein That Is Modified in a Cell Cycle–dependent Manner

David B. Friedman, Holly A. Sundberg, Eugene Y. Huang, and Trisha N. Davis

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Abstract. Spc110p (Nuf1p) is an essential component of the yeast microtubule organizing center, or spindle pole body (SPB). Asynchronous wild-type cultures contain two electrophoretically distinct isoforms of Spc110p as detected by Western blot analysis, suggesting that Spc110p is modified in vivo. Both isoforms incorporate $^{32}P_i$ in vivo, suggesting that Spc110p is post-translationally modified by phosphorylation. The slower-migrating 120-kD Spc110p isoform is converted to the faster-migrating 112-kD isoform after incubation with protein phosphatase PP2A, and specific PP2A inhibitors block this conversion. Thus, additional phosphorylation of Spc110p at serine and/or threonine residues gives rise to the slower-migrating 120-kD isoform. The 120-kD isoform predominates in cells arrested in mitosis by the addition of nocodazole. However, the 120-kD isoform is not detectable in cells grown to stationary phase (G0) or in cells arrested in G1 by the addition of α-factor. Temperature-sensitive cell division cycle (*cdc*) mutations demonstrate that the presence of the 120-kD isoform correlates with mitotic spindle formation but not with SPB duplication. In a synchronous wild-type population, the additional serine/threonine phosphorylation that gives rise to the 120-kD isoform appears as cells are forming the mitotic spindle and diminishes as cells enter anaphase. None of several sequences similar to the consensus for phosphorylation by the Cdc28p ($cdc2^{p34}$) kinase is important for these mitosis-specific phosphorylations or for function. Carboxy-terminal Spc110p truncations lacking the calmodulin binding site can support growth and are also phosphorylated in a cell cycle–specific manner. Further truncation of the Spc110p carboxy terminus results in mutant proteins that are unable to support growth and now migrate as single species. Collectively, these results provide the first evidence of a structural component of the SPB that is phosphorylated during spindle formation and dephosphorylated as cells enter anaphase.

THE centrosome is an essential organelle of eukaryotic cells, serving as the microtubule organizing center responsible for producing both cytoplasmic and nuclear microtubules. In the yeast *Saccharomyces cerevisiae*, centrosome function is provided by the spindle pole body (SPB)[1], an essential organelle involved in nuclear fusion as well as the formation of the mitotic and meiotic spindles necessary for chromosome segregation (Byers and Goetsch, 1974, 1975).

Ultrastructural analysis of wild-type SPBs shows that this organelle is composed of three distinct electron-dense layers or plaques. The central plaque is embedded in the nuclear envelope and the inner and outer plaques are associated with nuclear and cytoplasmic microtubules, respectively (reviewed by Rose et al., 1993; Winey and Byers, 1993). SPB duplication is coincident with initiation of DNA synthesis and bud formation in wild-type cells (Byers and Goetsch, 1974). The SPBs separate after DNA synthesis to form a short spindle in preparation for segregating the chromosomes at mitosis. Analysis of cell division cycle (*cdc*) mutants shows that these SPB events are orchestrated by an elaborate control mechanism that also governs bud formation and DNA synthesis (Byers and Goetsch, 1974, 1975). However, the cell cycle controls for these processes are separable, since SPB function, DNA synthesis and bud formation can be uncoupled by *cdc* mutations.

Using monoclonal antibodies raised against purified SPBs, Rout and Kilmartin (1990) determined the suborganellar localization of several SPB components. The 110-kD Spc110p localizes to the region spanning the central and inner plaques of the SPB. Spc110p contains a large central coiled-coil domain which serves as a spacer between the amino- and carboxy-terminal globular domains that are thought to reside in the central and inner plaques (Kilmartin et al., 1993). Strains dependent on mutant Spc110ps missing up to 82% of the central coiled-coil region are viable and produce SPBs with correspondingly shorter distances between the central and inner plaques. In addition, segments of the central coiled-coil region puri-

Address correspondence to Trisha N. Davis, Dept. of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195. Fax: (206) 685-1792.

1. *Abbreviations used in this paper*: SPB, spindle pole body; *cdc*, cell division cycle.

Table I. Strains Used in This Study

Strain name	Genotype	Source or reference
CRY1	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Fuller
DFY1	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 SPC110-201*	This study
DFY3	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 SPC110-202*	This study
HSY2-12C	*MATa ade2-1^{oc} ade3Δ can1-100 his3-11,15 leu2-3,112 lys2Δ::HIS3 spc110Δ::TRP1-1 ura3-1*	This study
JGY44-2A Sup407	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cmd1-1 SPC110-407*	Geiser et al., 1993
JGY44-2A Sup412	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cmd1-1 SPC110-412*	Geiser et al., 1993
JGY44-2A Sup419	*MATa ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cmd1-1 SPC110-419*	Geiser et al., 1993
9065-12-4	*MATa cdc4-1 leu2 ura3-52 hom3 his7*	B. Jensen
#245	*MATa GAL10-CLB2 clb1::URA3 clb2::LEU2 clb3::TRP1 clb4::HIS3 ade2-1^{oc} can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Fitch et al., 1992
RM14-3A	*MATa cdc7-1 bar1 ura3-52 trp1-289 leu2-3,112 his6*	McCarroll and Fangman, 1988
2785-41A	*MATa cdc14-1 his7 leu2 can1 ura3 hom3 sap3*	L. Hartwell
2786-27A	*MATa cdc15-2 his7 leu2 can1 ura3 hom3 sap3*	L. Hartwell

fied from *E. coli* produce rod-like structures in vitro with dimensions expected for a coiled-coil.

SPC110 was also identified in a screen for nuclear antigens and named *NUF1* (Mirzayan et al., 1992). Antibodies raised against Spc110p (Nuf1p) detect a 100-kD protein in mammalian cells, suggesting that this protein is evolutionarily conserved (Mirzayan et al., 1992). The amino-terminal globular domain contains several putative consensus sequences for phosphorylation by the Cdc28p kinase (Mirzayan et al., 1992), but the phosphorylation status of these sites has not been determined. The carboxy-terminal globular domain contains a consensus sequence for calmodulin binding (Geiser et al., 1993; Stirling et al., 1994), and carboxy-terminal Spc110p sequences bind calmodulin in vivo and in vitro (Geiser et al., 1993; Stirling et al., 1994; unpublished results). Dominant mutations in *SPC110* suppress defects in chromosome segregation displayed by the temperature-sensitive calmodulin mutant *cmd1-1* (Geiser et al., 1993). The interaction between Spc110p and calmodulin is required for the proper assembly of SPB components (Sundberg et al., 1996).

In this paper we show that Spc110p is phosphorylated in a cell cycle–dependent manner. Moreover, the presence and timing of Spc110p phosphorylation during the cell cycle correlate with the presence of the mitotic spindle apparatus. Spc110p represents the first example of a structural centrosome component that is modified by phosphorylation in a regulated manner consistent with a mitosis-specific function.

Materials and Methods

Media, Strains, and Genetic Manipulations

Liquid media were YPD (Geiser et al., 1991), YPD–P_i (Warner, 1991), or SD–URA (Sherman et al., 1986). Strains used in this study are listed in Table I. All strains are W303 background except 9065-12-4, 2785-41A, and 2786-27A, which are A364-A. Strains DFY1 (*SPC110-201*) and DFY3 (*SPC110-202*) were constructed by integration of EcoRI-linearized plasmids pDF38 and pDF37, respectively, into strain CRY1 by the two-step gene replacement strategy (Boeke et al., 1987). The *SPC110* alleles integrated into these strains are deleted for different amounts of the coiled-coil domain (see below), and can support growth as the sole source of *SPC110*. Replacement of wild-type *SPC110* sequences with those of the coiled-coil deletion alleles was indicated by the unique mobility of Spc110-201p or by the absence of signal from Spc110-202p on Western blots. Both strains exhibited growth rates that were indistinguishable from CRY1. Strain HSY2-12C is derived from the diploid HSY2 (Geiser et al., 1993), and contains a precise chromosomal deletion of *SPC110*. Plasmids containing various *SPC110* alleles were introduced into strain HSY2-12C by plasmid shuffle as described (Geiser et al., 1993). Plasmid transformations were carried out by the LiOAc method essentially as described (Ito et al., 1983).

Plasmids

Plasmids used in this study are listed in Table II. *SPC110* alleles that are deleted for portions of the central coiled-coil domain were constructed as follows. Plasmid pDF9 was constructed by removing sequences between the SmaI and EcoRV sites in the multiple cloning site of plasmid pBluescriptII KS$^+$ by cutting and subsequent ligation. A 2.5-kb HindIII fragment internal to the *SPC110* open reading frame (Genbank accession number Z11582) from pJG138 was then cloned into the HindIII site of pDF9, creating plasmid pDF12. To construct *SPC110-201*, sequences between the BglII site and NdeI site of the *SPC110* HindIII fragment in pDF12 were removed, creating plasmid pDF28. This was accomplished by restriction cutting at both sites, filling in using the Klenow fragment of DNA polymerase, and ligation of the resulting blunt ends. The *SPC110* HindIII fragment (containing the internal deletion) from pDF28 was then used to replace the wild-type *SPC110* HindIII fragment of pJG138, creating plasmid pDF29. The *SPC110-201* allele in plasmid pDF29 contains an in-frame internal deletion of 834 bp between the two destroyed enzyme sites. The *SPC110-202* allele was similarly constructed in pDF12 by removing the sequences between the BglII site and the second EcoRV site. This created plasmid pDF13, which contains an in-frame internal deletion of 1,314 bp between the two destroyed enzyme sites, thereby removing those sequences used to generate epitopes for raising anti-Spc110p antibodies (Geiser et al., 1993). The HindIII fragment from pDF13 was then used to replace the wild-type HindIII fragment in pJG138, creating plasmid pDF25.

Plasmids pDF37 and pDF38 are yeast integration vectors carrying either *SPC110-202* or *SPC110-201*, respectively. They were constructed by replacing the AlwNI fragments of either pDF29 or pDF25 (containing *CEN6 ARSH4*) with an AlwNI fragment derived from the yeast integrating vector pRS306.

A truncation of Spc110p at residue 828 was constructed as follows. *SPC110* contains two BclI sites, the second of which overlaps with codon 828. One of the two BclI sites in plasmid pHS29 was cleaved by partial digestion with BclI, and the resulting linear fragments were treated with the Klenow fragment of DNA polymerase to fill in the ends. An oligonucleotide containing a BamHI site (GGGATCCC) was then ligated to the blunt-end linear fragments, and the recircularized molecules were isolated in bacteria. Plasmids containing the linker were digested with BamHI to remove tandem insertions of the linker, and recircularized by ligation. This procedure resulted in two plasmids, pHS30 and pHS32, which con-

Table II. Plasmids Used in This Study

Plasmid	Parent plasmid	Relevant markers*	Source or reference
pBluescriptII KS+		f1 origin	Stratagene
pQE30		6xHIS tagged cloning vector	Qiagen
YEp24		2μ origin, *URA3*	Genbank Accession no. L09156
pRS316		*CEN4 ARSH4 URA3*, f1 origin	Sikorski and Hieter, 1989
pRS306		*URA3*, f1 origin	Sikorski and Hieter, 1989
pTD87	pJG138	1.5-kb BglII-SspI internal *SPC110* fragment	This study
pGF29	pRS306	2μ origin (YEp24 EcoRI fragment) inserted at AatII site	G. Zhu
pHS29	pRS316	*SPC110*	Sundberg et al., 1996
pHS30	pHS29	*SPC110* containing a BamHI 8-mer inserted in-frame at the first BclI site in the open reading frame	This study
pHS31	pHS29	*SPC110*	This study
pHS32	pHS29	*SPC110* containing a BamHI 8-mer inserted in-frame at the BclI site overlapping codon 828	Sundberg et al., 1996
pJG115	pGF29	*SPC110*	J. Geiser
pJG138	pRS316	*SPC110* contained on a PvuII-XbaI fragment cloned into a filled HindIII site and the XbaI site	J. Geiser
pJG141	pRS316	*spc110-213* (truncation at residue 756)	Geiser et al., 1993
pDF9	pBluescriptII KS+	f1 origin	This study
pDF12	pDF9	2.5-kb HindIII internal *SPC110* fragment	This study
pDF13	pDF12	1.2-kb HindIII internal *SPC110-202* fragment	This study
pDF19	pHS31	*SPC110-205* (S36A)	This study
pDF20	pHS31	*SPC110-206* (S91A)	This study
pDF21	pHS31	*SPC110-208* (S36A, S91A)	This study
pDF25	pJG138	*SPC110-202* (1.3-kb deletion of coiled-coil region)	This study
pDF28	pDF12	1.6-kb HindIII internal *SPC110-201* fragment	This study
pDF29	pJG138	*SPC110-201* (0.8-kb deletion of coiled-coil region)	This study
pDF31	pHS31	*SPC110-204* (T18A)	This study
pDF33	pHS31	*SPC110-207* (S116A)	This study
pDF34	pDF21	*SPC110-211* (T18A, S36A, S91A, S116A)	This study
pDF37	pRS306	*SPC110-202* (1.3-kb deletion of coiled-coil region)	This study
pDF38	pRS306	*SPC110-201* (0.8-kb deletion of coiled-coil region)	This study
pDF39	YEp24	*spc110-213* (truncation at residue 756)	This study
pDF41	pHS32	*spc110-214* (truncation at residue 828)	This study
pDF42	pHS31	*SPC110-212* (T840A, T847A, S853A)	This study

* All markers from the parent plasmid are present in the new construct.

tain a unique BamHI site inserted into either the upstream or downstream BclI site, respectively. pHS32 has been described previously (Sundberg et al., 1996). Both plasmids were able to support growth as the sole source of *SPC110*, and produced both Spc110p isoforms. Plasmid pDF41 was created from pHS32 by restriction cutting at the unique BamHI site, filling in the sticky ends using the Klenow fragment of DNA polymerase, and ligating the resulting blunt ends. This procedure produced a frame-shift at codon 828, resulting in the addition of six novel amino acids (GIDPDQ) followed by a stop codon. A similar truncation was made from plasmid pHS30, which contains the BamHI linker in the first BclI site. In this case, the frame-shift was made at the remaining BclI site in a similar fashion as above, and resulted in a stop codon only two novel amino acids after residue 828. Both versions of this truncation produced similar results. For simplicity, we only show data using the first construct contained on pDF41.

Plasmid pDF39 is a high copy number vector encoding a truncation of Spc110p at residue 756. It was constructed by replacing the AlwNI fragment of pJG141 (containing *CEN6 ARSH4*) with the AlwNI fragment derived from the yeast expression vector YEp24.

SPC110 alleles containing serine to alanine or threonine to alanine mutations were created by site-directed mutagenesis as described (Kunkel et al., 1987). This mutagenesis was carried out using plasmid pHS31, which contains *SPC110* with an NcoI site engineered into the initiating methionine codon and removed from the *URA3* gene by site-directed mutagenesis (*URA3* lacking the NcoI site was provided by M. Moser, Department of Biochemistry, University of Washington). Plasmids pDF31, pDF19, pDF20, and pDF33 carry the single-mutant alleles *SPC110-204* (T18A), *-205* (S36A), *-206* (S91A), and *-207* (S116A), respectively. Plasmid pDF42 carries the triple mutant allele *SPC110-212* (T840A, T847A, S853A). Plasmid pDF34, which carries the quadruple mutant allele *SPC110-211* (T18A, S36A, S91A, S116A), was created from a similar mutagenesis in which plasmid pDF21, which carries *SPC110-208* (S36A, S91A), was used as a starting template. The identity of each missense mutation was confirmed by DNA sequence analysis (Tabor and Richardson, 1987; reagents from United States Biochemicals Corp., Cleveland, OH).

Plasmid pTD87 contains a portion of the central coiled-coil region of *SPC110* fused to a 6xHIS tag and was used for the affinity purification of anti-Spc110p antibodies. pTD87 was constructed by inserting the BglII-SspI fragment of *SPC110* from pJG138 into the BamHI and SmaI restriction sites of pQE30, a 6xHIS fusion expression vector (Qiagen, Inc., Chatsworth, CA).

α-Factor, Nocodazole, and CLB-depletion Arrests

α-Factor arrests were performed on asynchronous cultures in early logarithmic growth by the addition of synthesized pheromone peptide (HHMI Biopolymer facility, University of Washington, Seattle, WA) to a final concentration of 6 μM, except for strain RM14-3A (*bar1*) when a final concentration of 200 nM was used. Strains were arrested in mitosis by the addition of nocodazole (Jacobs et al., 1988) at a final concentration of 5 μg/ml. Cultures were incubated in the presence of α-factor or nocodazole for approximately 1.5 generations, at which time the efficacy of the arrests was ascertained by bud morphology. Depletion of Clb2p in the *clb1Δ*, *clb2Δ*, *clb3Δ*, *clb4Δ*, *GAL10-CLB2* strain (no. 245) was performed as described (Fitch et al., 1992).

Cytological Techniques

Cell morphology was assayed using an Olympus BH-2 compound light microscope, and 100–200 cells were counted for each test point using a Petroff-Hausser counting chamber. Cells were prepared for flow cytometry as described (Muller, 1991), and the data were analyzed for DNA content as described (Method A, Slater et al., 1977). Cells were prepared for immunofluorescence as described (Davis, 1992), except that the primary antibodies were rat anti–α-tubulin ascites YOL1/34 at a dilution factor of 1:200, and the secondary antibodies were goat-anti–rat IgG conjugated to FITC (Bio-Rad Labs., Richmond, CA) at a dilution factor of 1:100. Stained cells were viewed using a Zeiss Axioplan microscope. Cells were prepared for thin-section electron microscopy as described (Byers and Goetsch, 1991), except that the fixative was 3% (wt/vol) glutaraldehyde in 50 mM phosphate buffer (pH 6.5) containing 0.5 mM $MgCl_2$. Serial sections were viewed using a Philips EM300 electron microscope.

Immunoblot Analysis

Total cell protein extracts were obtained by precipitation of whole cells with 10% (vol/vol) trichloroacetic acid (TCA) as described (Wright et al., 1989) unless otherwise indicated. Proteins were separated by 6% SDS-PAGE as described (Laemmli, 1970) unless otherwise indicated.

For Western blot analysis, electrophoretically separated protein extracts were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) using a Bio-Rad Trans-Blot Semi-Dry Transfer Cell for 25 min at 3–5 mA/cm^2, and the blotted membranes were blocked for >90 min with 10% nonfat milk in 25 mM Tris buffer (pH 7.5) containing 150 mM NaCl and 0.05% Tween-20. Blots were then incubated overnight in a 1% nonfat milk solution containing a 1:1,000 dilution of affinity-purified Spc110p polyclonal antibodies (see below). Immunoblots were developed using goat anti–rabbit IgG conjugated with horseradish peroxidase (1:5,000 dilution; Bio-Rad) and the ECL chemiluminescence system from Amersham, and the signal was detected with Hyperfilm-MP autoradiography film (Amersham Corp., Arlington Heights, IL).

The method of TCA precipitation used for the preparation of whole cell extracts in this study preserves both Spc110p isoforms well. Stirling et al. (1994) have also been able to preserve both isoforms using extracts made by glass bead lysis and boiling in SDS buffer. Mirzayan et al. (1992) only observed one Spc110p isoform, presumably due to the spheroplast-lysing method of protein extraction used by these authors, which included long incubations at 37°C during which time degradation of the 120-kD isoform might have occurred.

Preparation of Soluble Spc110p

Spc110p is largely insoluble under a variety of extraction conditions (Mirzayan et al., 1992). However, we were able to partially solubilize Spc110p by glass bead lysis in the presence of 20 mM Tris buffer (pH 8), 2 mM EDTA, 0.1 mM DTT and protease inhibitors. We suspect that differences in solubility arise from the pH of the solutions used (pH 6.5 buffers were used by Mirzayan et al., 1992). Protease inhibitors were diluted to a final concentration of 1 mM PMSF, 10 μM benzamidine, and 0.5 μg/ml each for antipain, leupeptin, pepstatin A and aprotinin. Cells were resuspended in 1 ml of lysis buffer (with protease inhibitors) and two volumes of glass beads. Cells were lysed by vortexing this mixture 20 times for 30 s separated by 30-s incubations on ice. The soluble fraction was obtained after centrifugation in a Sorval Microspin 24 microcentrifuge (12.3 *g* RCF).

Immunoprecipitation of ^{32}P-labeled Spc110p

Radiolabeled cells were washed in 1 ml cold H_2O and resuspended in 400 μl RIPA buffer (150 mM $CaCl_2$, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM EDTA, 1% NaDOC, 1% NP-40, 0.1% SDS, and the protease inhibitors described above). Cells were then lysed using one volume of glass beads by vortexing 10 times for 30 s separated by 30-s incubation on ice. The lysate was collected and centrifuged at 4°C in a Sorval Microspin 24 microcentrifuge (12.3 *g* RCF), and the supernatant was pre-cleared with 145 μl of de-fined Pansorbin cells (Calbiochem-Novabiochem, La Jolla, CA) for 2 h on ice. After centrifugation at 6,000 rpm for 5 min, each pre-cleared supernatant was incubated with 5 μl of affinity-purified polyclonal anti-Spc110p antibodies (see below) for 1 h on ice unless otherwise indicated. Each sample was then incubated for an additional 30 min on ice with 50 μl of de-fined pansorbin cells, followed by centrifugation through a 0.5 ml 10% sucrose RIPA cushion at 3,000 rpm for 10 min. The pelleted immune complexes were then washed three times in RIPA buffer and resuspended in 50 μl of SDS-PAGE sample buffer and boiled for 5 min before separation by 6% SDS-PAGE. Dried gels were analyzed using a Molecular Dynamics PhosphorImager model 400S (using 176 micron pixel size; Sunnyvale, CA) and ImageQuant software, and the resulting files were printed on a dye sublimation printer.

Affinity Purification of Anti-Spc110p Antibodies

Polyclonal antibodies 761H were raised against a GST fusion to the internal EcoRV fragment, containing amino acids 422-705 of Spc110p (Geiser et al., 1993). Antibodies were then affinity purified against a 6xHis tagged fusion protein of a larger, overlapping BglII-SspI fragment containing amino acids 265-757 of Spc110p. The fusion protein, encoded on plasmid pTD87, was expressed in GM1 *E. coli* for 3 h at 37°C after induction with 1 mM IPTG at 30 Klett units. Cells were harvested by centrifugation and lysed in a French press (19,000 psi) in the presence of 1 mM PMSF, 100 μg/ml RNase A, and a flake of lyophilized DNase I was then added. Soluble lysate was loaded on to Ni-NTA resin (Qiagen) and washed extensively with a 50 mM phosphate buffer (pH 6.0) containing 1 M NaCl and 10% glycerol. The fusion protein was eluted from the column with a pH gradient (pH 6.0–2.0) in 50 mM phosphate buffer containing 300 mM NaCl and 10% glycerol. Pooled fractions were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry electroblotting as described above. The membrane was stained with Ponceau S (Sigma Chem. Co., St. Louis, MO) and the 60-kD fusion protein was excised. Antibodies were affinity purified as described (Pringle et al., 1989) with some modification. Briefly, antiserum was incubated with the 60-kD membrane-bound protein. The membrane was washed with 25 mM Tris buffer (pH 7.5) containing 150 mM NaCl, and 0.05% Tween-20. Finally, the antibodies were eluted with 100 mM glycine (pH 2.2) and neutralized with 0.1 vol 1 M Tris buffer (pH 8.0).

The specificity of the affinity-purified anti-Spc110p antibodies used in the experiments presented here is excellent and indistinguishable from that using a mixture of Spc110p monoclonal antibodies (35A11 and 45D10; Rout and Kilmartin, 1990). Moreover, the affinity-purified antibodies detect no signal above background on Western blots of DFY3 lysates containing Spc110-202p, which lacks the antigenic region of the central coiled-coil domain.

Results

Spc110p Is Phosphorylated at Serine/Threonine Residues

Affinity-purified polyclonal antibodies raised against a central portion of Spc110p (Materials and Methods) detect two electrophoretically distinct Spc110p isoforms on Western blots of wild-type protein lysates prepared from an asynchronous culture (Fig. 1 *A*, lane *1*). Two major isoforms are evident, one migrating with an apparent molecular mass of 120 kD and the other migrating with the expected molecular mass of 112 kD. Both Spc110p isoforms incorporate radiolabeled $^{32}P_i$ in vivo (Fig. 1 *B*, lane *1*), suggesting that Spc110p is posttranslationally modified by phosphorylation.

The addition of mammalian PP2A, a serine/threonine-specific phosphatase, to a soluble extract containing Spc110p resulted in the conversion of the 120-kD Spc110p isoform into the 112-kD isoform (Fig. 1 *A*, lane *2*). Moreover, this conversion was blocked by the addition of the PP2A inhibitors okadaic acid and microcystin (Fig. 1 *A*, lane *3*). The inhibition by okadaic acid and microcystin was overcome by a 10-fold increase in PP2A (Fig. 1 *A*, lane *4*). Conversion of the 120-kD isoform was a direct effect of the exogenous phosphatase, since we observed the same conversion when extracts were incubated on ice in the presence of a nonspecific phosphatase isolated from the Arctic shrimp *Pandalus borealis* (United States Biochemicals;

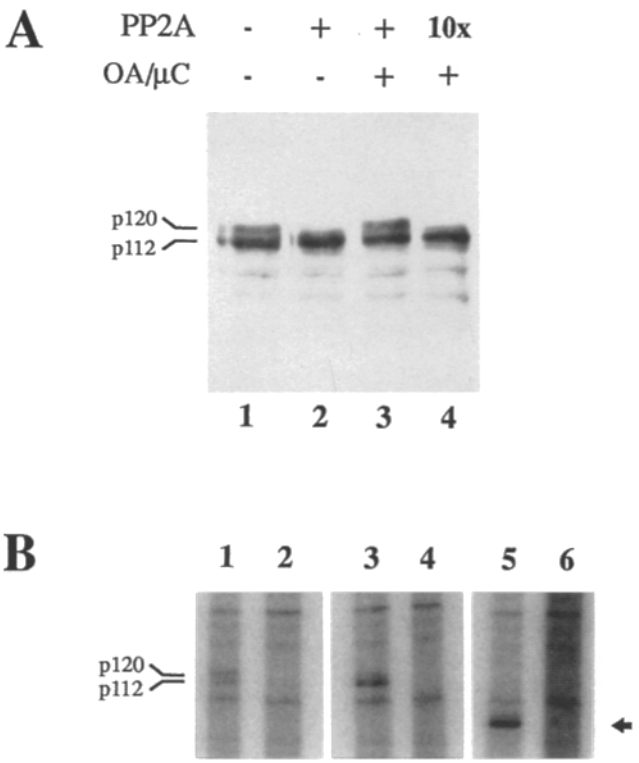

Figure 1. Spc110p is phosphorylated. (*A*) Sensitivity of Spc110p modification to bovine serine/threonine-specific phosphatase PP2A. Spc110p was partially solubilized from a 25-ml culture of strain CRY1 (100 Klett units) as described in Materials and Methods. Western blot analysis was performed as described in Materials and Methods. 10 μl of the soluble Spc110p extract was loaded on an SDS-PAGE gel for each of the following conditions. Lane *1*, Soluble extract containing Spc110p incubated at 30°C for 40 min before addition of SDS-PAGE sample buffer and boiling. Lane *2*, Same as lane *1* except that 10 units of Bovine protein phosphatase PP2A (prepared from beef hearts; gift of J. Campbell, N. Ahn, and E. Krebs, Howard Hughes Medical Institute, Department of Pharmacology and Biochemistry, University of Washington) in a solution containing protease inhibitors (Materials and Methods) was added. Lane *3*, Same as lane *2* except that okadaic acid (*OA*) and microcystin (*μC*) were added to a final concentration of 10 nM. Lane *4*, Same as lane *3* except that 100 units (10×) of PP2A was added. (*B*) Immunoprecipitation of $^{32}P_i$-labeled Spc110p. Cultures of strain DFY3 harboring various *SPC110*-containing plasmids were grown in SD–URA to 40 Klett units, washed twice in 5 ml YPD–P_i, and diluted to 20 Klett units in 20 ml YPD–P_i at 30°C. When cultures had grown to approximately 100 Klett units, ^{32}P-labeled orthophosphoric acid (DuPont New England Nuclear, Boston, MA) was added to a final concentration of 150 μCi/ml to 2 ml of each culture. Cultures were incubated with label for 60 min at 30°C, and Spc110p was immunoprecipitated from each culture as described in Materials and Methods. Lane *1*, pJG138 (CEN *SPC110*). Lane *2*, pJG138, but no primary antibody was added to the immunoprecipitation. Lane *3*, pJG115 (2μ *SPC110*). Lane *4*, YEp24 (2μ). Lane *5*, pDF39 (2μ *spc110–213*). Lane *6*, pJG141 (CEN *spc110-213*). 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated. The arrow on the right indicates the mobility of the 756 truncation.

data not shown). Endogenous yeast enzymes are not expected to be active on ice. Thus, the 120-kD Spc110p isoform results from additional serine/threonine phosphorylation of the 112-kD isoform. The nature of the ^{32}P signal

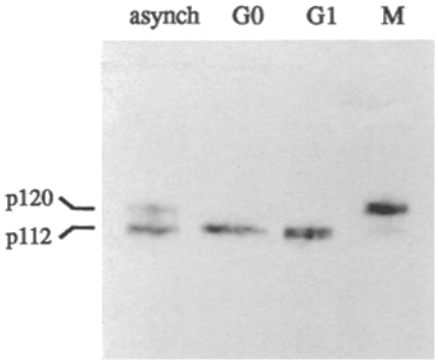

Figure 2. Cell cycle–specific presence of Spc110p phosphorylation. Western blot analysis was performed as described in Materials and Methods on protein extracts from wild-type strain CRY1 grown at 30°C under the following conditions: *asynch*, asynchronous culture in early logarithmic phase of growth; *G0*, cells grown to stationary phase (>48 h); *G1*, cells arrested by addition of α-factor (100% shmoo morphology); *M*, cells arrested in mitosis by the addition of nocodazole (79% large-budded cells). Aliquots representing extract from approximately 4×10^6 cells were loaded for each lane. 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated.

arising from the 112-kD isoform is not known. In some gels, the lower, 112-kD isoform resolved into two tightly spaced isoforms (data not shown). The presence of this tight doublet was not apparent in every experiment and was not studied further.

The 120-kD Isoform Accumulates in Mitotic Cells

To investigate the possibility that the serine/threonine phosphorylation in the 120-kD isoform of Spc110p is cell cycle regulated, we monitored Spc110p by Western blot analysis from cells that were arrested at different stages of the cell cycle. Cells grown to stationary phase (G0) or arrested in G1 by the addition of the mating pheromone α-factor did not contain the 120-kD isoform (Fig. 2). In contrast, cells arrested in mitosis by the addition of nocodazole, which destabilizes microtubules, accumulated the 120-kD isoform almost exclusively (Fig. 2).

The steady state level of Spc110p increased in cells expressing Spc110p from a high copy number plasmid as judged both by in vivo $^{32}P_i$ incorporation (Fig. 1 *B*, lane *3*) and by Western blot analysis (Fig. 3). However, the majority of the extra signal was found only in the lower, 112-kD isoform, with the 120-kD isoform present at almost equivalent levels to those observed in isogenic cells dependent on Spc110p expression from a low copy number plasmid. Cultures overproducing Spc110p still arrested in mitosis in response to nocodazole, but the conversion of Spc110p into the 120-kD isoform was incomplete (Fig. 3). Thus, an unknown mechanism limits the serine/threonine phosphorylation that gives rise to the 120-kD isoform to a subset of the total population of Spc110p, but the level of $^{32}P_i$ incorporation and protein production of the lower, 112-kD isoform is not limiting.

Mutations Blocking Progression through the Cell Cycle Define a Window for Production of the 120-kD Isoform

Strains carrying several different *cdc* mutations were used

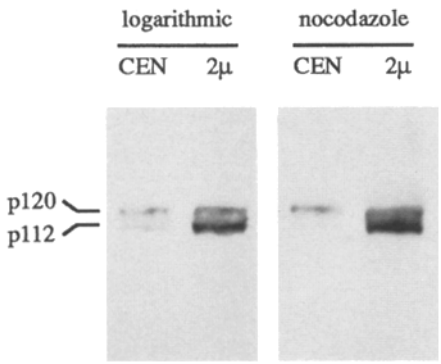

Figure 3. Overexpression of *SPC110*. Western blot analysis was performed as described in Materials and Methods on protein extracts from logarithmic and nocodazole-arrested cultures of strain HSY2-12C dependent upon *SPC110* expression from either a low copy number plasmid or a high copy number plasmid. Logarithmic cultures were grown at 30°C to 100 Klett units, and extract from approximately 4×10^6 cells was loaded for each logarithmic sample. After the logarithmic samples had been taken, the remainder of each culture was arrested by the addition of nocodazole (86–89% large budded cells), and an equal volume of extract was loaded for each nocodazole-arrested sample. (*CEN*) *SPC110* on low copy number plasmid pJG138. (*2μ*) *SPC110* on high copy number plasmid pJG115. 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated.

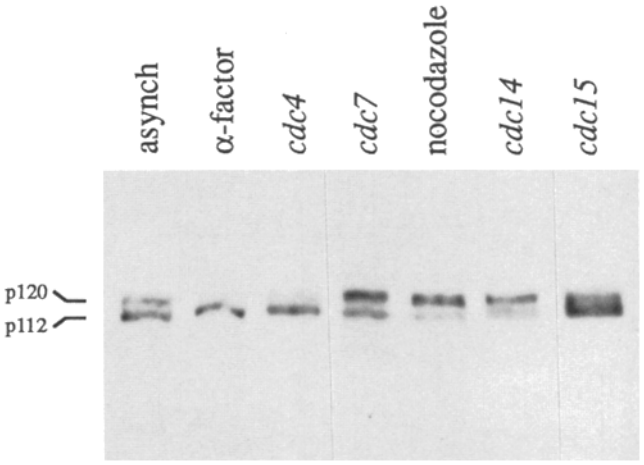

Figure 4. Pattern of Spc110p phosphorylation at different *cdc* arrests. Western blot analysis was performed as described in Materials and Methods on extracts obtained from cells arrested at various stages of the cell cycle specific for the indicated *cdc* mutations. 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated. (*asynch*) Asynchronous culture of *cdc4* strain grown at room temperature. (α-factor) *cdc4* strain arrested with α-factor at room temperature (95% shmoo morphology). (*cdc4*) *cdc4* cells were incubated for 4 h at 36°C and displayed 91% large or multiple-elongated buds and 1N DNA content. (*cdc7*) *cdc7* cells were first synchronized in G1 by the addition of α-factor, collected by filtration and released into fresh medium at the restrictive temperature. Cells were then incubated for 2 h at 36°C and displayed 93% medium or large budded cells and 1N DNA content. (*nocodazole*) *cdc4* cells were arrested with nocodazole at room temperature (90% large budded cells). (*cdc14*) *cdc14* cells were incubated for 2 h at 36°C and displayed 98% large budded cells and 2N DNA content. 81% of cells also contained two distinct DAPI-staining centers. (*cdc15*) *cdc15* cells were incubated for 2 h at 36°C and displayed 96% large budded cells and 2N DNA content. 66% of cells also contained two distinct DAPI-staining centers. Aliquots representing extract from approximately 4×10^6 cells were loaded for each lane.

to define further the stages of the cell cycle during which the 120-kD isoform is present. Cells arrested in G1 by the temperature-sensitive *cdc4* mutation did not contain significant amounts of this isoform (Fig. 4). Mitotic spindles were not evident by tubulin immunofluorescence in these *cdc4*-arrested cells, consistent with previously published electron microscopy showing the presence of duplicated but unseparated SPBs at a *cdc4* arrest (Byers and Goetsch, 1974).

In contrast, the 120-kD isoform accumulated in cells arrested by the *cdc7* mutation at the G1/S boundary (Fig. 4). Tubulin immunofluorescence revealed the presence of a short mitotic spindle in 93% of the cells at the *cdc7* arrest. Cells arrested by the *cdc14* mutation also accumulated the 120-kD isoform, and cells arrested by *cdc15* exhibited a heterogeneous population of Spc110p isoforms (Fig. 4). The majority of the large budded cells at the *cdc14* and *cdc15* arrests contained separated nuclei, indicating a mitotic arrest point later than that for nocodazole-arrested cells in which the chromosomes do not segregate (Jacobs et al., 1988). All of the *cdc* strains tested exhibited the same G1- and mitosis-specific Spc110p isoforms described for wild-type strain CRY1 when arrested with α-factor or with nocodazole under permissive conditions (data shown only for *cdc4* strain, Fig. 4).

Serine/Threonine Phosphorylation of Spc110p Accumulates with the Formation of the Mitotic Spindle and Disappears as Cells Enter Anaphase

The two Spc110p isoforms were monitored in a synchronous wild-type culture released from a G1 α-factor arrest. The levels of the two isoforms clearly alternated with each other during the two cell cycles examined (Fig. 5). Significantly, the serine/threonine phosphorylation that gives rise to the 120-kD isoform appeared as the spindle was formed and was removed as the cells entered anaphase. As examined by immunofluorescence, the proportion of cells with a pre-anaphase spindle directly correlated with the proportion of the 120-kD isoform in the culture (Fig. 5). Spindle formation occurred as cells entered G2, consistent with previously published studies demonstrating that spindle formation occurs after DNA synthesis (Byers and Goetsch, 1974).

Our analysis of spindle formation was confirmed by electron microscopy in a parallel experiment. At a time early in the cell cycle when 50% of Spc110p was present as the 120-kD isoform, 64% of cells ($n = 36$) exhibited very short but complete spindles, with the remainder of the cells containing duplicated side-by-side SPBs. This result is not significantly different from expected if the presence of the 120-kD isoform correlates with the presence of a short spindle ($\chi^2 = 2.78$; $P = 0.10$). In conjunction with our finding that the 120-kD isoform is not present at a *cdc4* arrest (Fig. 4), these results demonstrate that production of the 120-kD isoform is not required for SPB duplication.

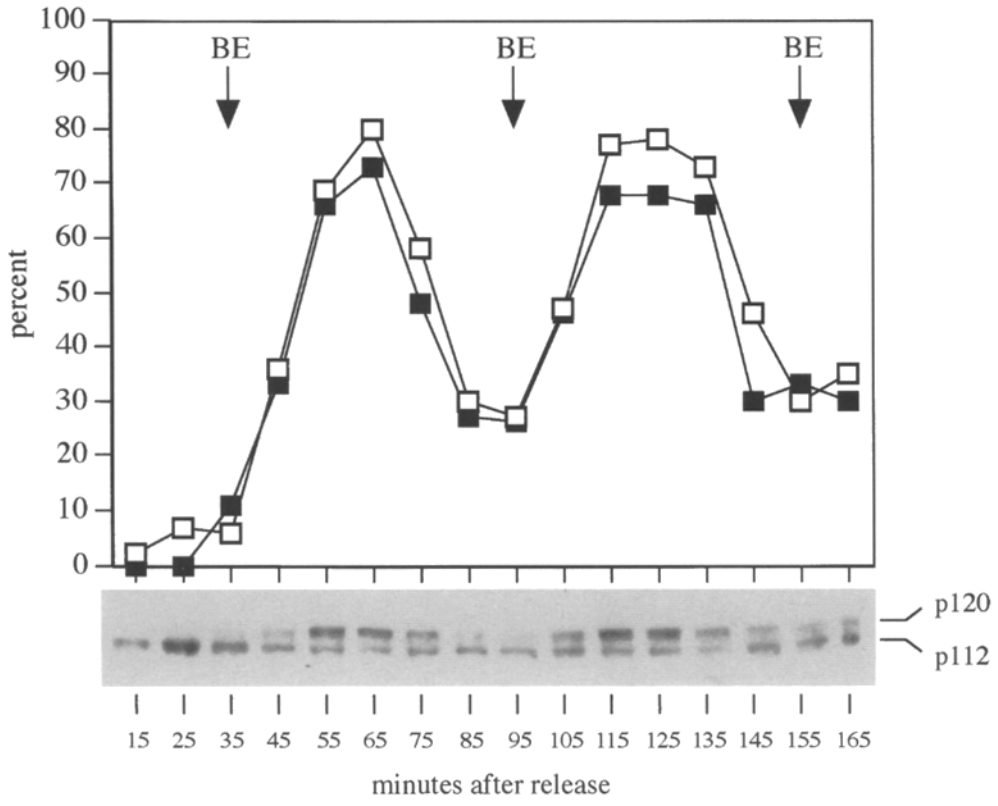

Figure 5. Pattern of Spc110p phosphorylation in a synchronous wild-type culture. A 25-ml culture of strain CRY1 (at 50 Klett units) in YPD medium was arrested in G1 by the addition of α-factor (Materials and Methods). Cells arrested in G1 (96% unbudded shmoo morphology) were released by collection onto a 0.8-μm filter disc (Millipore Corp., Bedford, MA) and washing with three culture volumes of pre-warmed fresh medium (30°C). Cells were resuspended in 25 ml of pre-warmed fresh medium and placed into a 30°C shaking water bath. The first time point was taken 15 min after cells were initially washed with fresh medium lacking α-factor. Additional time points were taken at 10-min intervals as indicated. Cells at each time point were collected for measurement of bud morphology, Spc110p modification, DNA content and microtubule staining pattern (Materials and Methods). BE indicates the time at which small buds first appear during each cycle. Shown is the percentage of phosphorylated Spc110p relative to total Spc110p (■) and the percentage of cells containing a mitotic spindle before anaphase (□). The shortest mitotic spindles appeared as brightly-staining centers of tubulin, sometimes associated with small rays of microtubules that were not stained as intensely. This staining pattern was distinct from that for cells earlier in the cell cycle, which exhibited a wider array of longer cytoplasmic microtubules, the intensity of which was no different from that of the common focus from which they emanated. Confirmation that these brightly staining centers of tubulin were short spindles was obtained by electron microscopy (see text). Cells with short spindles or with long spindles and a continuous region of DAPI staining material are included in □. Cells exhibiting long spindles associated with two distinct DAPI-staining centers indicative of cells in the anaphase or telophase stages of mitosis are not included. Shown below is the Western blot analysis demonstrating the alternation of the 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms throughout the experiment. The film used to generate this figure was scanned and analyzed using NIH Image software to calculate the percentage of phosphorylated Spc110p relative to total Spc110p shown in the graph (■). Aliquots representing extract from ~1.5 × 10^6 cells were loaded for each lane.

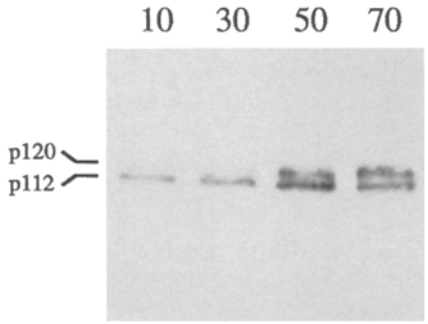

Figure 6. Pattern of Spc110p phosphorylation in a synchronous culture arrested by the depletion of Clb2p in the absence of Clb1p, Clb3p, and Clb4p. Western blot analysis was performed as described in Materials and Methods. Strain no. 245 was grown at 30°C to 65 Klett units, synchronized with α-factor (92% unbudded shmoo morphology) and released to the *CLB*-depletion arrest as described (Fitch et al., 1992). The time (in minutes) that each sample was taken after cultures were released into fresh YPD is indicated above each lane. 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated. Approximately 50% of the cells had progressed through S-phase by 30 minutes after release, and progression through S-phase was virtually complete by the 50-min time point. As expected from published results, at the 70-min time point, 84% of the cells exhibited a single elongated bud.

Production of the 120-kD Isoform is Independent of CLB1, CLB2, CLB3, and CLB4 Activity

B-cyclins *CLB1-CLB4* are essential for spindle formation but not for SPB duplication (Fitch et al., 1992). We determined whether the serine/threonine phosphorylation of Spc110p that gives rise to the 120-kD isoform occurs in a strain deleted for *CLB1*, *CLB2*, *CLB3*, and *CLB4* and maintained by constitutive expression of *CLB2* (Fitch et al., 1992). We monitored the Spc110p isoforms in cells released from an α-factor arrest under conditions that depleted cells of Clb2p (Fitch et al., 1992).

Upon depletion of Clb2p, these cells arrested with an accumulation of the 120-kD Spc110p isoform (Fig. 6). Approximately half of the Spc110p was in the 120-kD isoform by 50 min after cells were released from α-factor, and once

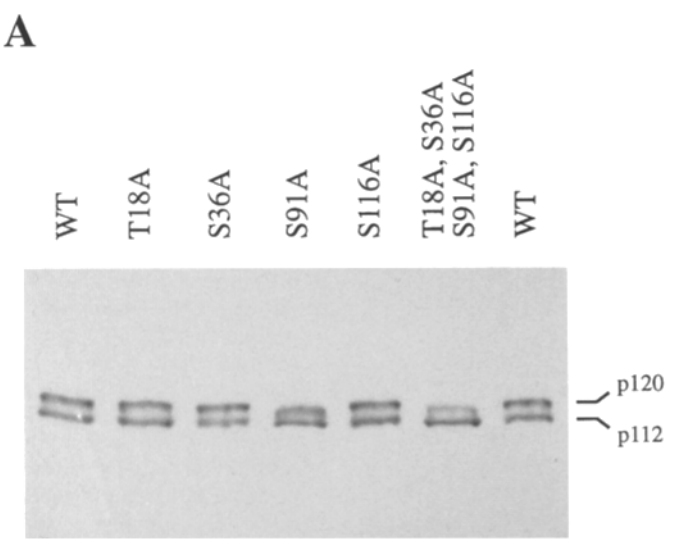

Figure 7. Phosphorylation state of Spc110p containing serine to alanine and threonine to alanine mutations from asynchronous 30°C cultures (at 100 Klett units). Western blot analysis was performed as described in Materials and Methods on extracts of strain HSY2-12C maintained by plasmids harboring the following *SPC110* alleles. (*A*) (*WT*) pHS31 (*SPC110*). (*T18A*) pDF31 (*SPC110-204*). (*S36A*) pDF19 (*SPC110-205*). (*S91A*) pDF20 (*SPC110-206*). (*S116A*) pDF33 (*SPC110-207*). (*T18A, S36A, S91A, S116A*) pDF34 (*SPC110-211*). (*B*) (*T840A, T847A, S853A*) pDF42 (*SPC110-212*). (*WT*) pHS31 (*SPC110*). 120- (*p120*) and 112-kD (*p112*) Spc110p isoforms are indicated.

established, this level remained constant for the remainder of the experiment (>3 h). The 120-kD isoform was not apparent at the 30-min time point when ~50% of the cells had completed DNA synthesis, indicating that the 120-kD isoform arises in these cells after S-phase as it does in wild-type cells. Short mitotic spindles were not evident by tubulin immunofluorescence after 1 h at the arrest, consistent with previously published electron microscopy showing the presence of duplicated but unseparated SPBs in cells held at this arrest for 1 h (Fitch et al., 1992). 30% of the cells held at the *CLB*-depletion arrest for more than 3 h were found to contain short spindles (also demonstrated by Fitch et al., 1992). However, the 120-kD isoform first appeared more than two hours before these spindles formed. Thus, in the absence of Clb1p-4p activity, the serine/threonine phosphorylation that gives rise to the 120-kD isoform is not sufficient to allow spindles to form.

The Serine/Threonine Phosphorylation Giving Rise to the 120-kD Isoform Does Not Occur at Canonical Cdc28p Phosphorylation Sequences

The Cdc28p kinase, along with the appropriate G1- or G2-specific cyclin cofactors, has been shown to phosphorylate targets essential for the entry into S-phase or mitosis, respectively (reviewed in Nasmyth, 1993). We tested whether any of the putative Cdc28p phosphorylation sequences located in the amino terminus of Spc110p (Mirzayan et al., 1992) represent the sites of Spc110p serine/threonine phosphorylation that give rise to the 120-kD isoform. We introduced mutations of serine to alanine or threonine to alanine into sequences within Spc110p that are similar to the S/TPXR/K consensus for Cdc28p phosphorylation (Langan et al., 1989; Shenoy et al., 1989). Of the three sites identified by Mirzayan et al. (1992), SPTK (residues 36–39) is a perfect match to this consensus, and the other two, SPR (residues 91–93) and SQPLK (residues 116–120), differ at one or more residues. A liberal search for any Spc110p sequence resembling this consensus (a serine or threonine residue followed by a proline residue) identified only one additional sequence, TPV (residues 18–20).

Yeast strains were able to survive with alleles of *SPC110* that carried various combinations of these mutations, including the quadruple mutant allele, as the sole source of *SPC110*. The growth rates of these strains were indistinguishable from that of CRY1. Furthermore, yeast strains supported by these mutant Spc110ps still produced two Spc110p isoforms (Fig. 7 *A*). The quadruple mutant was phosphorylated in the same G1- and mitosis-specific manner as wild-type Spc110p when cells were arrested with α-factor or with nocodazole (data not shown). Both isoforms of the quadruple mutant exhibited a slightly altered mobility which was also observed for the isoforms of the single mutant S91A (Fig. 7 *A*). Since removal of the 120-kD isoform by PP2A treatment does not result in a similar altered mobility of the lower, 112-kD wild-type isoform (Fig. 1 *A*), it is likely that the change in mobility of the quadruple mutant results from an alteration in the protein structure caused by the S91A mutation. Alternatively, S91 may represent one of many residues that, when phosphorylated, collectively give rise to the 120-kD isoform. Thus, the four residues tested here do not represent essential sites of phosphorylation and, with the possible exception of S91, do not contribute to the formation of the 120-kD isoform in wild-type extracts.

Internal Deletions and Carboxy-Terminal Truncations of Spc110p Map Regions Necessary for Production of the 120-kD Isoform

To identify regions of Spc110p that are necessary for the serine/threonine phosphorylation that gives rise to the 120-kD isoform, we monitored Spc110p in strains carrying carboxy-terminal truncations of Spc110p (Geiser et al., 1993). Full-length Spc110p contains 944 residues. Strains

dependent on Spc110-412p, Spc110-407p, and Spc110-419p, which are truncated at residues 881, 863, and 856, respectively, are all viable and exhibit both isoforms in asynchronous cultures (Fig. 8). We also constructed and tested an in-frame internal deletion of Spc110p that was missing a portion of the central coiled-coil domain (Spc110-201p). A strain dependent on Spc110-201p for growth was viable and produced two isoforms in asynchronous cultures (Fig. 8). Strains expressing any of these four variant proteins exhibited G1- and mitosis-specific isoforms analogous to those seen for the wild-type strain when arrested with α-factor or with nocodazole (data not shown).

We also monitored the isoform production of additional Spc110p truncations previously shown to be unable to support growth as the sole source of Spc110p (Geiser et al., 1993). Protein production of these nonfunctional truncations was assayed in strain DFY3, which is maintained by a variant form of Spc110p (Spc110-202p) that is not detected by the antibodies used in this study (Materials and Methods). A strain expressing a nonfunctional Spc110p truncation at residue 828 in the carboxy-terminal globular domain was found to produce only one species (Fig. 8). Another nonfunctional truncation at residue 756, near the end of the coiled-coil domain, was also found to produce only one species (Fig. 8). Both non-functional truncations migrated with an apparent molecular mass similar to the predicted values for these truncations (98 and 89 kD for the 828 and 756 truncations, respectively). In addition, we occasionally observed a tight doublet for both the 756 and the 828 truncation analogous to the tight doublet sometimes observed with the lower, 112-kD isoform of the wild-type protein (data not shown). As a control for Spc110p modification in DFY3, both the 112- and 120-kD isoforms were produced when this strain was transformed with a plasmid expressing *SPC110* (data not shown). These results delineate a region of Spc110p between residues 828 and 856 that either contains the phosphorylated residues giving rise to the 120-kD isoform, or contains sequences essential for this phosphorylation to occur elsewhere in the polypeptide.

To determine whether the region between residues 828 and 856 contains the phosphorylated residues giving rise to the 120-kD isoform, we replaced the two threonine and one serine residue in this region with alanine residues by site-directed mutagenesis (Materials and Methods). The resulting triple mutant allele *SPC110-212* (T840A, T847A, S853A) was able to support growth as the sole source of Spc110p at a rate indistinguishable from that of CRY1. Western blot analysis showed that both isoforms were still present for this triple mutant (Fig. 7 *B*). Thus, these three

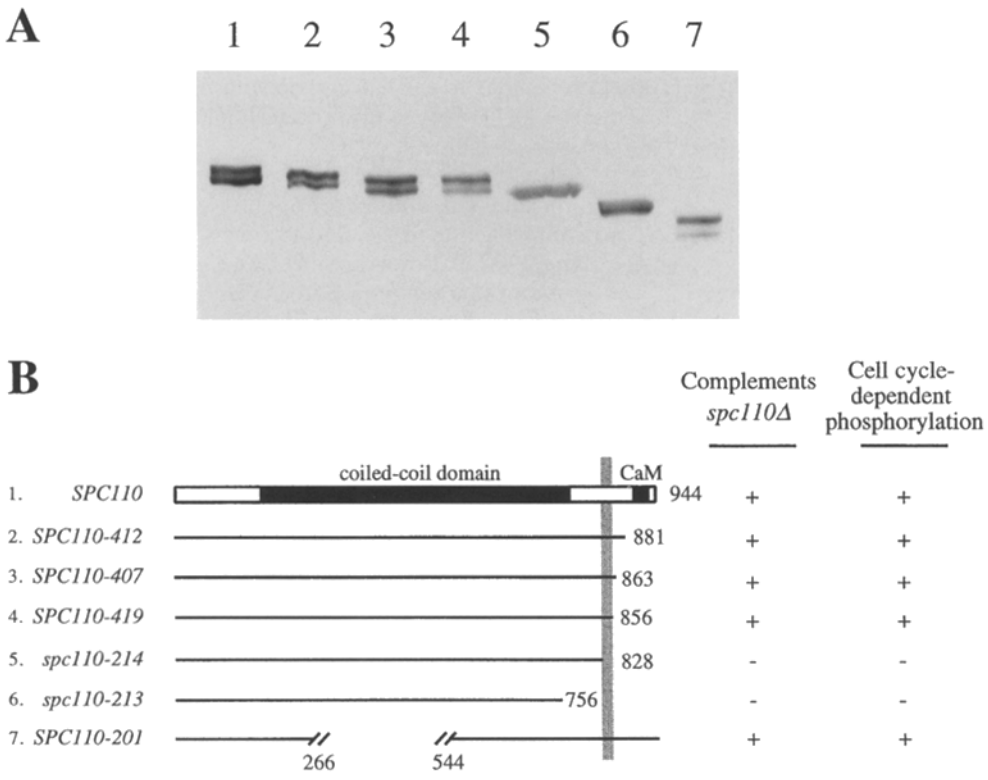

Figure 8. Phosphorylation state of various Spc110p truncations and in-frame internal deletions from asynchronous cultures. (*A*) Western blot analysis of extracts from strains containing the following proteins after separation by SDS-PAGE using a 4–8% acrylamide gradient (stabilized by a 5–12% sucrose gradient). Lane *1*, wild-type strain CRY1 (944 residues). Lane *2*, Spc110p-412p (881 residues) from strain JGY44-2A Sup412. Lane *3*, Spc110-407p (863 residues) from strain JGY44-2A Sup407. Lane *4*, Spc110p-419p (856 residues) from strain JGY44-2A Sup419. Lane *5*, Spc110-214p (truncation at residue 828) from strain DFY3 carrying plasmid pDF41. Lane *6*, Spc110-213p (truncation at residue 756) from strain DFY3 carrying plasmid pJG141. Lane *7*, Spc110-201p (666 residues), which contains an internal in-frame deletion of a portion of the central coiled-coil domain, from strain DFY1. An equal volume of extract representing approximately 4×10^6 cells was loaded for each lane except for lanes *5* and *6*, where approximately four times and two times as much was loaded, respectively. (*B*) Graphical representation of the Spc110p truncations and deletions shown in *A* with respect to certain features of the full-length protein (944 residues). The coiled-coil domain lies between residues 168 and 773 (Kilmartin et al., 1993). The calmodulin binding site is located between residues 900 and 914 (Stirling et al., 1994). Horizontal lines indicate regions that are left intact by the truncated and deleted proteins, and the numbers next to them indicate the residue number at the truncation/deletion end-points. The boxed region between residues 828 and 856 delineates a region of the protein that is necessary for Spc110p phosphorylation as described in the text. The category labeled cell cycle–dependent phosphorylation refers to the presence of a mitosis-specific isoform exhibiting a mobility shift analogous to the mobility shift observed for the wild-type 120-kD isoform.

residues do not represent essential sites of phosphorylation and do not contribute to the formation of the 120-kD isoform in wild-type extracts.

We investigated several additional properties of the non-functional truncations at residues 828 and 756 to understand better the nature of the defect in these proteins. Both non-functional truncations are expressed in cells, albeit at reduced levels (Fig. 8). Overexpression of the 756 truncation was still insufficient to support growth as the sole source of Spc110p. However, overexpression of this truncation did result in increased steady state levels of both protein production and in vivo $^{32}P_i$ incorporation for this truncation (data shown only for $^{32}P_i$ incorporation, Fig. 1 *B*, lane *5*). This same increase was found for the lower, 112-kD isoform of the wild-type protein, but not for the 120-kD isoform (Figs. 1 *B* and 3). Thus, in this regard the 756 truncation behaves in an analogous manner to the lower, 112-kD isoform of Spc110p.

Finally, we performed immunofluorescence studies on these truncations to determine if they were properly localized to the SPB. We were unable to detect the 756 or the 828 truncation at the SPB or elsewhere in cells of strain DFY3 even though the wild-type protein was clearly localized to the SPB in this strain (data not shown). However, these negative findings could have been a consequence of the low abundance of the truncated proteins in DFY3. Overexpression of the 756 truncation resulted in a general nuclear staining pattern which was also observed for overexpressed wild type (data not shown). Thus, the 756 truncation is properly localized to the nucleus, but the high background of nuclear staining prohibited us from determining whether or not the 756 truncation is incorporated into the SPB.

Discussion

We report that the 110-kD component of the *S. cerevisiae* SPB, Spc110p, is phosphorylated in a cell cycle–dependent manner. Specific serine/threonine phosphorylation of Spc110p gives rise to a slower-migrating 120-kD isoform. In a synchronous culture, the 120-kD isoform appears as the mitotic spindle is formed and disappears as cells enter anaphase. DNA synthesis is completed before this phosphorylation of Spc110p occurs. The level of the 120-kD isoform in response to drugs or a *cdc4* or *cdc7* mutation confirms that this isoform appears as the spindle forms. This isoform is not present in cells arrested with a single SPB by α-factor or in cells arrested by a *cdc4* mutation with duplicated SPBs and no spindle. This isoform is present in cells arrested by a *cdc7* mutation with short spindles and in cells blocked before anaphase by nocodazole. The presence of the 120-kD isoform is indicative of SPBs that are competent to form a spindle rather than spindle formation per se, since spindles are absent at the nocodazole arrest.

Interestingly, the 120-kD isoform accumulated when cells were blocked by the removal of Clb1p-Clb4p (Fig. 6) even though the duplicated SPBs were not separated by a mitotic spindle. As was noted at the nocodazole arrest, the accumulation of the 120-kD isoform at the *CLB*-depletion arrest may mark SPBs that are competent to undergo mitotic spindle formation. In this case, however, spindle formation cannot commence without the activation of processes that are dependent on *CLB1-4* activity. The 120-kD isoform also accumulated in cells arrested by a *cdc14* mutation even though the chromosomes had segregated (Fig. 4). Many of the *cdc* mutations uncouple SPB function, DNA synthesis and bud formation at the arrest (Byers and Goetsch, 1974, 1975). The *cdc14* cells may inappropriately transit anaphase without completing all anaphase functions (including Spc110p dephosphorylation) at the restrictive temperature. Indeed, *cdc14* cultures exhibit high chromosome loss rates at high temperatures and become inviable after a few hours of incubation at the restrictive temperature (Hartwell and Smith, 1985; Palmer et al., 1990; W. Raymond, personal communication).

The fact that the 120-kD isoform is not present at the *cdc4* block but is present in cells depleted of Clb1p-Clb4p suggests that the serine/threonine phosphorylation giving rise to this isoform is dependent on Clb5p or Clb6p. Both of these B-cyclins are intact in the strain deleted for *CLB1-CLB4*, but Clbp-Cdc28p kinases are not active in cells arrested at the *cdc4* block (Schwob et al., 1994). Depletion of the six known B-cyclins (Clb1p-6p) results in a *cdc4* phenotype (Schwob et al., 1994). Furthermore, the *cdc4* defect can be suppressed by overexpression of *CLB4* (Bai et al., 1994), *CLB2* or *CLB5* (Jensen, B., and B. Byers, personal communication). It is unlikely that either Clb5p-Cdc28p or Clb6p-Cdc28p are directly responsible for the phosphorylation of Spc110p studied here, since mutation of the threonine and serines in all of the sites that are similar to the Cdc28p consensus phosphorylation sequence has no effect on protein function or production of the 120-kD isoform.

A deletion analysis demonstrated that the region between residues 828 and 856 of Spc110p is required for the production of mitosis-specific isoforms analogous to the wild-type 120-kD isoform. Since the two threonines and one serine in this region are not essential for function or for the production of the 120-kD isoform, it seems likely that this region of the polypeptide is essential for the serine/threonine phosphorylation to occur elsewhere in the polypeptide. For example, this region could contain part of a binding site for the protein kinase(s) responsible for the serine/threonine phosphorylation. Alternatively, this region of Spc110p could contain an SPB-localization signal, and Spc110p may only be phosphorylated in this manner when properly localized to the SPB. Our finding that the amount of the 120-kD isoform does not increase significantly when the protein is overproduced (Fig. 3) could be explained by either of these models, since the kinase could be limited in its activity or in its ability to bind Spc110p, and the number of Spc110p molecules that can assemble into the SPB is likely to be limited.

The timing during the cell cycle of the serine/threonine phosphorylation specific to the 120-kD isoform is very similar to the appearance of centrosomal phosphoepitopes recognized by the monoclonal antibody MPM-2 in both mammalian cells (Vandré and Borisy, 1989) and in *Schizosaccharomyces pombe* (Masuda et al., 1992). However, Spc110p does not contain any sequences that match the consensus recognition sequences for MPM-2 (Westendorf et al., 1994), and MPM-2 antibodies do not detect either Spc110p isoform in Western blots (unpublished results).

Additional evidence from a variety of organisms suggests that phosphorylation of certain substrates is necessary for spindle formation to occur, and removal of these or other phosphorylations is necessary for progression through anaphase. In *Drosophila melanogaster*, mutations in the aurora serine/threonine kinase arrest cells with duplicated but unseparated centrosomes (Glover et al., 1995). The mitosis-specific Human Plk1 protein kinase, which shares homology with the *S. cerevisiae* Cdc5p and *D. melanogaster* polo serine/threonine kinases, localizes to the spindle poles of mammalian cells up to metaphase and then redistributes to the midzone as cells transit anaphase (Golsteyn et al., 1995). Mammalian cells can also be reversibly arrested at a stage before anaphase onset by the addition of okadaic acid, a specific serine/threonine phosphatase inhibitor (Vandré and Wills, 1992). In *S. cerevisiae*, mutations in the serine/threonine phosphatase PP1 lead to a similar block at the metaphase/anaphase transition (Hisamoto et al., 1994; Black et al., 1995; MacKelvie et al., 1995). Our observations of the timing of Spc110p phosphorylation during mitosis implicates Spc110p as a target of these or analogous kinases and phosphatases in yeast.

In summary, we have shown that the yeast 110-kD spindle pole body component Spc110p is modified by serine/threonine phosphorylation as the mitotic spindle forms. We also show that this phosphorylation is removed as cells enter anaphase. This is the first evidence of a structural centrosomal or SPB component that is modified by phosphorylation at serine or threonine residues in a cell cycle–dependent manner. We have mutated the obvious potential sites of Spc110p phosphorylation and found them not to affect Spc110p function or production of the 120-kD isoform. Determining the exact nature and location of the phosphorylated residues in Spc110p will lead to a better understanding of how these modifications contribute to the cell cycle regulation of SPB function in yeast, but may be hampered by the low abundance and relative insolubility of Spc110p in yeast.

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