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Spc98p and Spc97p of the yeast γ-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p

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Previously, we have shown that the yeast γ-tubulin, Tub4p, forms a 6S complex with the spindle pole body components Spc98p and Spc97p. In this paper we report the purification of the Tub4p complex. It contained one molecule of Spc98p and Spc97p, and two or more molecules of Tub4p, but no other protein. We addressed how the Tub4p complex binds to the yeast microtubule organizing center, the spindle pole body (SPB). Genetic and biochemical data indicate that Spc98p and Spc97p of the Tub4p complex bind to the N-terminal domain of the SPB component Spc110p. Finally, we isolated a complex containing Spc110p, Spc42p, calmodulin and a 35 kDa protein, suggesting that these four proteins interact in the SPB. We discuss in a model, how the N-terminus of Spc110p anchors the Tub4p complex to the SPB and how Spc110p itself is embedded in the SPB.

Keywords: centrosome/γ-tubulin complex/Spc110p/yeast/spindle pole body

Introduction

Tubulin is a heterodimer composed of α- and β-tubulin that assembles to form hollow cylinders known as microtubules. In many cell types, microtubule assembly is initiated at distinct sites (microtubule nucleation sites) at the so-called microtubule organizing centers (MTOCs). Pickett-Heaps (1969) coined this generic term to collectively define the microtubule nucleating activity of the morphologically distinct centrosomes, basal bodies, spindle pole bodies (SPBs) and nucleus-associated bodies (reviewed by Joshi, 1994).

How the structurally diverse MTOCs fulfill their common microtubule organizing function became clearer with the discovery of γ-tubulin as a probably universal component of microtubule nucleation sites. γ-tubulin was first identified as a suppressor of a temperature-sensitive β-tubulin mutation in the fungus *Aspergillus nidulans* (Weil *et al.*, 1986; Oakley and Oakley, 1989; Oakley *et al.*, 1990). Since then γ-tubulin has been discovered in many different organisms, including human, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis* and plant cells (Stearns *et al.*, 1991; Zheng *et al.*, 1991; Horio and Oakley, 1994; Liu *et al.*, 1994; Lopez *et al.*, 1995; Sunkel *et al.*, 1995). Recently, a second γ-tubulin gene has been identified in *Drosophila* (Tavosanis *et al.*, 1997).

Studies using electron microscopy have shown that centrosomes consist of a pair of centrioles surrounded by a protein mass called the pericentriolar material, that exhibits the microtubule nucleating activity (Gould and Borisy, 1977) and contains γ-tubulin (Stearns *et al.*, 1991; Moudjou *et al.*, 1996). Structural characterization of centrosomes from early *Drosophila* embryos by EM tomography identified γ-tubulin in numerous ring complexes which may represent microtubule nucleation sites (Moritz *et al.*, 1995a,b). Structurally similar γ-tubulin-containing complexes with a sedimentation coefficient of 25S were also identified in the cytoplasm of human cells and *Xenopus* eggs (Stearns and Kirschner, 1994; Zheng *et al.*, 1995). Their subsequent purification from frog eggs revealed seven proteins including α-, β- and γ-tubulin. The additional proteins had apparent molecular weights of 195, 133, 109 and 75 kDa. This purified γ-tubulin complex appears in the electron microscope as an open ring structure with a diameter of 25–28 nm. It has the capability to bind to microtubule ends and to initiate microtubule polymerization from tubulin subunits (Zheng *et al.*, 1995).

In yeast *Saccharomyces cerevisiae* γ-tubulin is encoded by the essential *TUB4* gene (Sobel and Snyder, 1996; Marschall *et al.*, 1996; Spang *et al.*, 1996a). Temperature-sensitive *tub4(ts)* mutants are either defective in the formation of microtubules at the newly formed SPB, while the mother SPB is not affected (Marschall *et al.*, 1996), or, as is the case for the *tub4-1* mutant, are impaired at the point of spindle formation (Spang *et al.*, 1996a). Components of the Tub4p complex were identified by genetic screenings. *SPC98*, which codes for the previously described 90 kDa SPB component (Rout and Kilmartin, 1990), was discovered as a dosage-dependent suppressor of *tub4-1* (Geissler *et al.*, 1996). Similarly, *SPC97* was found as a suppressor of a *spc98(ts)* mutant (Knop *et al.*, 1997). Analysis of temperature-sensitive *spc98(ts)* and *spc97(ts)* mutants revealed phenotypes similar to those shown for the *tub4-1(ts)* allele (Geissler *et al.*, 1996; Knop *et al.*, 1997). In addition, *spc97-20* showed a defect in SPB duplication (Knop *et al.*, 1997). In agreement with their role in microtubule organization, Tub4p, Spc98p and Spc97p were associated with the inner and outer plaques of the SPB (Rout and Kilmartin, 1990; Spang *et al.*, 1996a; Knop *et al.*, 1997), the substructures that organize the cytoplasmic and nuclear microtubules respectively (Byers and Goetsch, 1975; Byers, 1981) (Figure 6A). Using genetic and biochemical techniques, it was shown that Tub4p, Spc98p and Spc97p mutually interact and that the three proteins are part of a 6S complex (Geissler *et al.*, 1996; Knop *et al.*, 1997).

In this study, we purified a yeast Tub4p complex and found Tub4p, Spc98p and Spc97p as its sole components. We addressed how this complex binds to the SPB. Our

analysis identified the N-terminal domain of Spc110p as the docking site for the Tub4p complex. This domain of Spc110p interacts with Spc98p and Spc97p, but not with Tub4p. Finally, it is shown that Spc110p is present in a complex with the SPB component Spc42p, calmodulin and a 35 kDa protein. Based on these results, we propose a model for the anchorage of the yeast γ-tubulin complex to the SPB.

Results

Composition and stoichiometry of the yeast γ-tubulin complex

Previously we have shown that Spc98p, Spc97p and Tub4p, the yeast γ-tubulin, co-immunoprecipitate and that they are part of a complex with a sedimentation coefficient of ~6S (Geissler *et al.*, 1996; Knop *et al.*, 1997). To further understand the composition of this complex, we used functional ProteinA–Spc98p (ProA–Spc98p) and Spc97p–ProteinA (three repeats of Protein A; Spc97p–3ProA) fusion proteins to purify such complexes by affinity purification over IgG columns. This approach resulted in the isolation of either Spc98p and Tub4p together with Spc97p–3ProA or Spc97p and Tub4p in complex with ProA–Spc98p. Spc98p species appeared as diffuse bands due to phosphorylation (G.Pereira, submitted) (Figure 1A). The identity of the isolated proteins was confirmed by immunoblotting (Figure 1B). ProA-tagged species were always detected due to the binding of the secondary antibody to the ProA tag. Other known SPB components, such as Kar1p, Spc110p (data not shown) and the tubulin subunits Tub1p (yeast α-tubulin) and Tub2p (yeast β-tubulin) were not present, even in substochiometric amounts (Figure 1C). An identical result for the composition of this complex was obtained by native immunoprecipitation of 3HA-tagged Spc97p from [^{35}S]methionine pulse-labeled cells. Only Spc98p and Tub4p were co-immunoprecipitated with 3HA–Spc97p (Figure 1D).

Previous results indicate that there is more than one molecule of Tub4p per complex (Knop *et al.*, 1997). In order to address this question for Spc97p and Spc98p, we used functional 3MYC- and 3HA-tagged variants of these proteins. We co-expressed *SPC97-3MYC* together with *SPC97-3HA* (an identical experimental setup was also used for *SPC98*). If the Tub4p complex contains two or more molecules of Spc97p, anti-HA antibodies should precipitate Spc97p-3HA as well as Spc97p-3MYC. However, if the complex contains only one molecule of Spc97p, the anti-HA antibodies should precipitate only Spc97p-

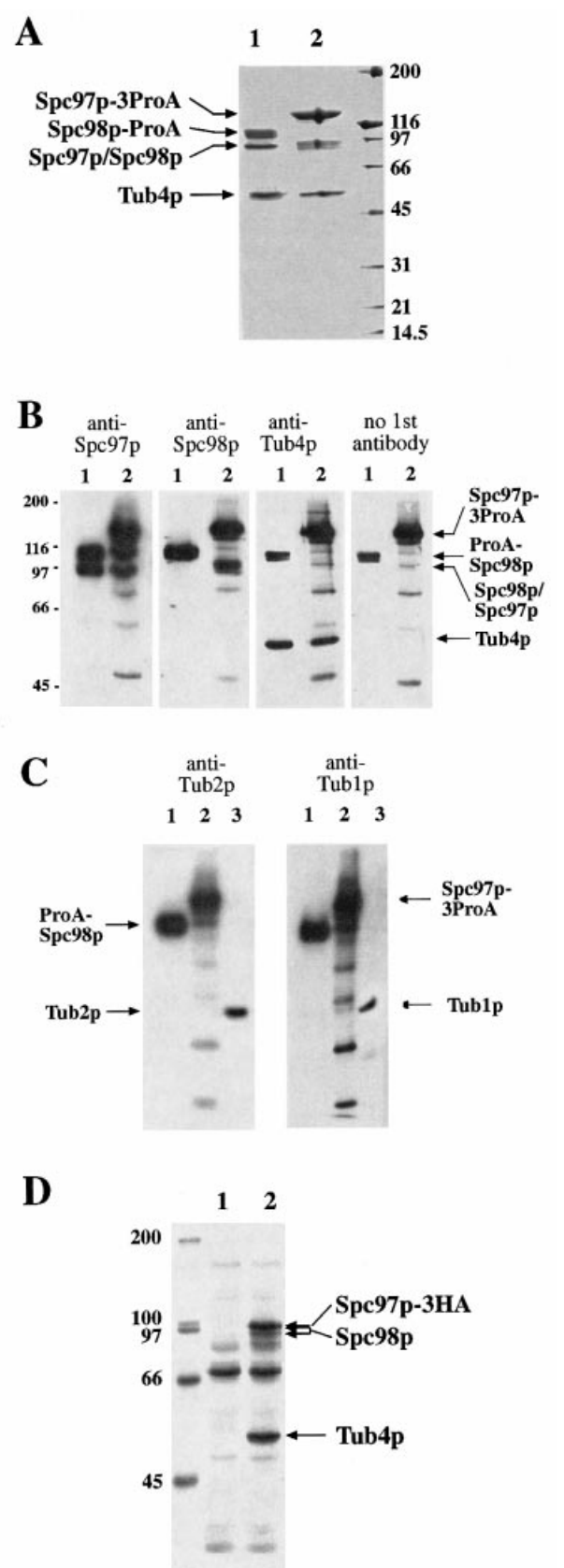

Fig. 1. Composition of the γ-tubulin complex of yeast *S.cerevisiae*. (**A**) Strains bearing functional ProteinA fusions with either *SPC98* (ESM282, lane 1; ProA–Spc98p) or *SPC97* (YMK47, lane 2; Spc97p–3ProA) were used to purify the γ-tubulin complex. The purified proteins were separated on an 8–18% SDS–PAGE gel which was then stained with Coomassie blue. (**B**) Western blotting and immunodetection of Spc98p, Spc97p and Tub4p in the protein isolates. Isolates from *ProA–SPC98* (lane 1) and of *SPC97–ProA* cells (lane 2). Antibodies are as indicated in the figure. A minor amount of Spc97–3ProA seems to be degraded. The degradation products can be identified by immunodetection with IgGs coupled to horseradish peroxidase (no 1st antibody). (**C**) The Tub4p complex of yeast does not contain α- or β-tubulin. Purified Tub4p complex from *ProA–SPC98* (lanes 1) or from SPC97–3ProA (lane 2) cells was probed with anti-Tub1p or anti-Tub2p antibodies. Purified SPBs were used as positive controls (lane 3). (**D**) Extracts of pulse-labeled YMK18 (*SPC97*; control, lane 1) and YMK22 (*SPC97-3HA*, lane 2) cells were subjected to immunoprecipitation under non-denaturing conditions using anti-HA antibodies.

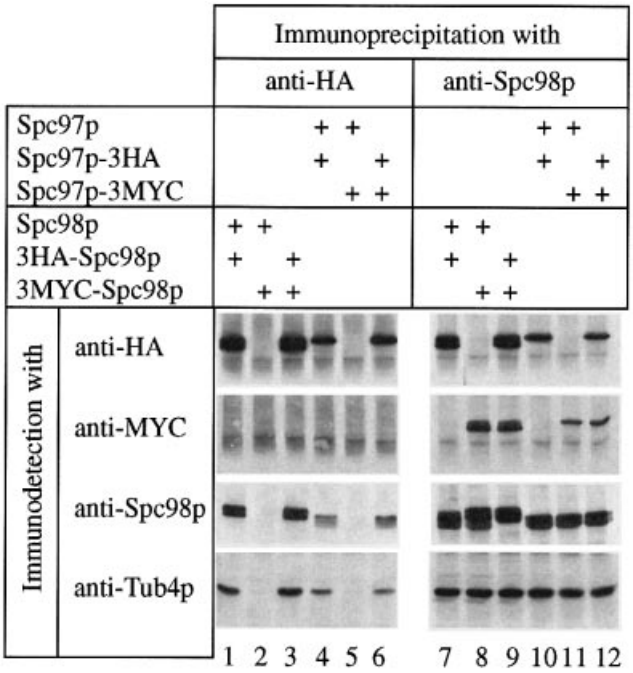

Fig. 2. Stoichiometry of Spc98p and Spc97p in the γ-tubulin complex. Cells of strains that express two of *SPC98*, *SPC98-3HA* or *SPC98-3MYC* in all possible combinations as indicated in the panel (lanes 1 and 7, strain YMK90; lanes 2 and 8, strain YMK91; lanes 3 and 9, strain YMK92), were lysed by vortexing with glass beads (Knop *et al.*, 1997). Lysates were used for immunoprecipitation with anti-HA or anti-Spc98p antibodies coupled covalently to protein G–Sepharose. The immunoprecipitates were solubilized in sample buffer and four identical blots were made and probed with the indicated antibodies. An identical experimental setup was used for *SPC97* (lanes 4 and 10, strain YMK93; lanes 5 and 11, strain YMK94; lanes 6 and 12, strain YMK95).

3HA but not Spc97p-3MYC. A similar approach has been used successfully to investigate the composition of the anaphase-promoting complex (Lamb *et al.*, 1994). After immunoprecipitation with anti-HA antibodies (12CA5), we assayed the immunoprecipitates for the presence of the 3MYC-tagged species. However, no MYC-tagged protein of either Spc98p or Spc97p could be co-immunoprecipitated with the 3HA-tagged variant (Figure 2, lanes 3 and 6, anti-MYC blot). Control immunoprecipitations using polyclonal anti-Spc98p antibodies confirmed the sensitivity of the detection as well as the presence of 3HA- and 3MYC-tagged proteins (Figure 2, lanes 7–12). Additional controls showed that the complexes were intact (anti-Tub4p blot) and that MYC- and HA-tagged species were in complexes (Figure 2, lanes 7–12). As described before, Tub4p was co-immunoprecipitated with Tub4p-3HA using anti-HA antibodies, indicating at least two molecules of Tub4p in the complex (Knop *et al.*, 1997; data not shown). These results suggest that only one molecule of Spc98p and Spc97p, but two or more molecules of Tub4p, are present in the yeast γ-tubulin complex.

Binding of the γ-tubulin complex to the SPB

γ-Tubulin complexes can also be found in the cytoplasm; however, they are in an inactive state. Therefore centrosomal proteins that dock the γ-tubulin complex to the SPB may regulate its activity as well as the number of microtubule nucleation sites. Using the yeast two-hybrid system, we investigated the interaction of Tub4p, Spc98p and Spc97p with other SPB proteins. One likely candidate was Spc110p, a filamentous protein that connects the central plaque with the inner plaque (Rout and Kilmartin, 1990; Kilmartin and Goh, 1996). The N-terminal domain of Spc110p is directed towards the inner plaque, while its C-terminus is embedded in the central plaque of the SPB (Figure 6A) (Spang *et al.*, 1996b; Sundberg *et al.*, 1996). Therefore, we speculated that the N-terminal domain of Spc110p may be the docking site for the yeast γ-tubulin complex at the inner plaque. Subdomains of Spc110p were fused to the Gal4-activator domain and two-hybrid interaction of these constructs were assayed with either Spc98p, Spc97p or Tub4p fused to the lexA DNA-binding domain. While the C-terminal domain (referred to as C-Spc110p) and the central domain (Z-Spc110p) of Spc110p showed no interaction (data not shown), the N-terminal domain of Spc110p (amino acids 1–204; Spc110p$^{1–204}$) revealed strong interaction with Spc98p and Spc97p, but not with Tub4p (Figure 3A). An identical result was obtained, when only amino acids 1–176 of Spc110p (Spc110p$^{1–176}$) were tested. Mutated versions of Spc97p (Knop *et al.*, 1997) showed weaker (Spc97-14p) or no (Spc97-20p) interaction with Spc110p$^{1–204}$ (Table I). The full-length Spc110p could not be tested in the two-hybrid system due to the severe toxic effects of overexpressed *Gal4-SPC110p*.

These results prompted us to test whether we can obtain Spc98p- and Spc97p-mediated interaction of Tub4p with Spc110p$^{1–204}$. This was indeed the case: when we co-overexpressed Spc97p and Spc98p from the strong *Gal1*-promoter together with *Gal4-SPC110*$^{1-204}$, we obtained a strong signal in the two-hybrid system with *lexA-TUB4* (Figure 3B), but not with *lexA-tub4-1*^{ts} or *lexA-Xgam* (Table I). This signal was dependent on the simultaneous expression of *SPC98* and *SPC97* (Figure 3B).

We then sought for additional, genetic evidence for an interaction of *SPC98* and *SPC97* with *SPC110*. We assayed for synthetic lethality of *spc110-2* (Kilmartin and Goh, 1996) when combined with *spc97(ts)*, *spc98(ts)* or *tub4-1*. Synthetic lethality is an indication for a functional relationship of two genes. *spc110-2* was synthetic-lethal in combination with temperature-sensitive alleles of *SPC98* (Geissler *et al.*, 1996) or *SPC97* (Knop *et al.*, 1997) in an allele-specific manner. In contrast, *tub4-1* (Spang *et al.*, 1996a) was not synthetic-lethal when combined with *spc110-2* (Table II). Further genetic evidence for an interaction of Spc110p$^{1–204}$ with the γ-tubulin complex came from overexpression experiments. Strong overexpression of *SPC110* $^{1-204}$ was toxic to cells. This toxicity was increased when *SPC110*$^{1-204}$ was co-overexpressed simultaneously with *Gal1-SPC98*, *Gal1-SPC97* and *Gal1-TUB4*. These *Gal1-SPC98*, *Gal1-SPC97* and *Gal1-TUB4* constructs were integrated into the genome and their simultaneous overexpression did not cause a growth defect (Figure 3C).

We looked for biochemical evidence for an interaction of the Tub4p complex with Spc110p$^{1–204}$. Immunoprecipitation experiments using anti-Spc110p, anti-Tub4p, anti-Spc98p or anti-Spc97p antibodies did not reveal a link between Spc110p and the Tub4p complex (data not shown). This failure may result from the insolubility of Spc110p (Mirzayan *et al.*, 1992; data not shown). To overcome

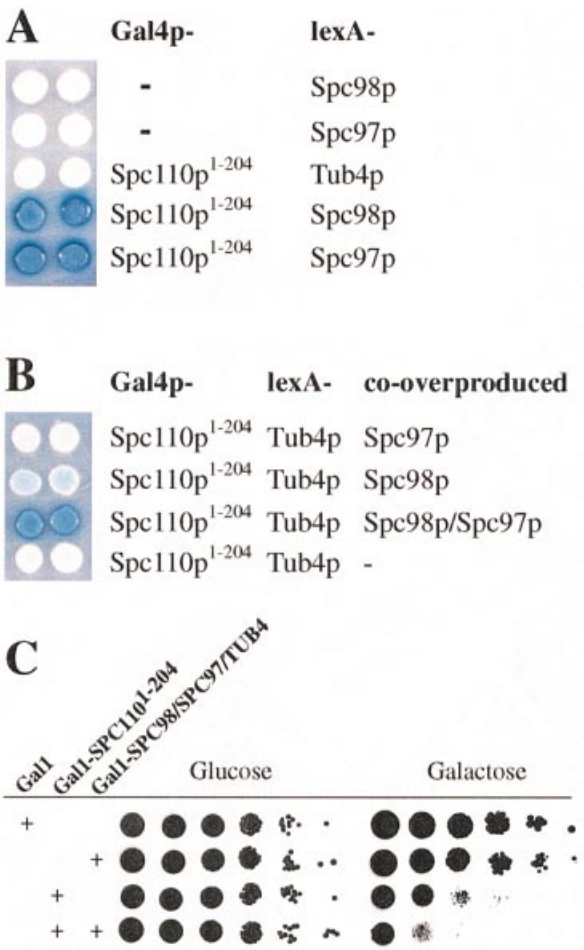

Fig. 3. Interaction of Spc110p with components of the γ-tubulin complex. (**A**) Two-hybrid interaction of Spc110p$^{1–204}$ with Spc98p, Spc97p or Tub4p. The empty plasmid was used as a control (–). (**B**) Interaction of Tub4p with Spc110p$^{1–204}$ in the presence of co-overproduced Spc98p and/or Spc97p. *SPC97* and/or *SPC98* were expressed under the control of the *Gal1*-promoter from *CEN-TRP1* plasmids harbouring one or both promoter fusions. (**C**) Overexpression of Spc110p$^{1–204}$ is toxic. *SPC110*$^{1–204}$ was expressed under the control of the *Gal1*-promoter either in wild type cells YPH499 or in cells of strain ESM387-3 (YPH499 containing integrated *Gal1-TUB4 Gal1-SPC98 Gal1-SPC97* constructs). Serial dilutions of cells were dropped on selective plates containing either glucose (repression) or galactose (induction) and growth was assayed for 4 days at 30°C.

this problem, we tested the binding of the Tub4p complex to GST-Spc110p$^{1–204}$ purified from *Escherichia coli* (Figure 4A). Binding of the Tub4p, Spc98p and Spc97p–3ProA was analyzed using immunoblotting, because these species are minor components in total yeast cell lysates. A fraction of the Tub4p complexes present in the crude cell lysate bound to GST-Spc110p$^{1–204}$. This binding was dependent on the presence of the intact N-terminal domain of Spc110p, since the binding to a mutated version of GST-Spc110p* (amino acids 3–176 of Spc110p and Y15C) was clearly reduced (Figure 4B to C). In agreement with this reduced binding, Spc110p* showed a 4-fold reduction in its interaction with Spc98p or Spc97p in the two-hybrid system compared with Spc110p$^{1–204}$ (Table I), although it was expressed equally to Spc110p$^{1–204}$ (data not shown). Furthermore, the Tub4p complex did not bind to the N-terminal domain of another coiled-coil protein of the SPB, Spc42p (Donaldson and Kilmartin, 1996; data not shown), nor did it bind to GST (Figure 4).

We tested whether isolated GST-Spc110p$^{1–204}$ from *E.coli* binds directly to the purified Tub4p complex. In this experiment, the Tub4p complex from *SPC97-3ProA* cells was purified on IgG-coupled latex microspheres with a diameter of 0.2 μm (Tub4p beads). Buffer and washing steps were similar to that used for the purification of the Tub4p complex by IgG–Sepharose (Figure 1). As a control, cell lysate from *SPC97* cells was incubated with the beads (control beads). The Tub4p and control beads were then incubated with the purified GST-Spc110p$^{1–204}$, followed by spinning the beads through a glycerol cushion to remove unbound material. The beads were collected and investigated by immunoblotting. Binding of GST-Spc110p$^{1–204}$ to Tub4p beads was detectable and this was 5- to 10-fold above the background of non-specific binding as determined with the control beads (Figure 4D). Taken together, our results suggest that Spc98p and Spc97p, but not Tub4p, interact directly with the N-terminal domain of Spc110p.

Anchorage of Spc110p to the SPB

Our results point to the N-terminal domain of Spc110p as the docking site for the Tub4p complex at the nuclear face of the SPB. To test how Spc110p itself is anchored in the SPB, we aimed to purify Spc110p complexes from cells producing a functional ProA–Spc110p fusion protein. In contrast to the Tub4p complex (Figure 1), ProA–Spc110p was in the high-speed pellet of yeast cell lysates (data not shown). This pellet was extracted with various buffers in order to determine the conditions for the solubilization of ProA–Spc110p-containing complexes. A buffer containing 1 M NaCl, 1% Triton X-100, EGTA and EDTA turned out to be most suitable. A subsequent purification step using an IgG column revealed the isolation of the ProA–Spc110p protein as well as at least three additional proteins with apparent molecular weights of 14.5 kDa, 35 kDa and 45 kDa (Figure 5A, lane 2, bands marked with an asterisk). These three proteins appear to be specifically associated with ProA–Spc110p, as they were not present in the proteins that bound non-specifically to the IgG column when similar incubations were performed with an extract from *SPC110* cells (Figure 5A, lane 1). The 14.5 kDa protein was identified by immunoblotting as Cmd1p, the yeast calmodulin (Figure 5A). This was not surprising, since it has been shown that calmodulin binds to a peptide within the C-terminal domain of Spc110p in a Ca^{2+}-independent manner (Geiser *et al.*, 1993; Stirling *et al.*, 1994; Spang *et al.*, 1996b). Using specific anti-Spc42p antibodies, the 45 kDa protein was shown to be Spc42p (Figure 5A). Spc42p is a component of the second intermediate layer (IL2) of the SPB, which is localized adjacent to the central plaque on the cytoplasmic side of the SPB (Donaldson and Kilmartin, 1996; Bullitt *et al.*, 1997). The identity of the 35 kDa protein is the subject of current investigation. Finally, we tested whether the Spc110p complex contains Spc98p, Spc97p, Tub1p, Tub2p, Tub4p or Kar1p. These proteins were not detected by immunoblotting using affinity purified antibodies while a strong signal was obtained with enriched SPBs (Figure 5B, and data not shown). When we performed immunoblots with enriched ProA–Spc110p, we

Table I. Two-hybrid interaction of the N-terminus of Spc110p with components of the Tub4p–tubulin complex

	β-Galactosidase activity (U)				Co-expression of
	Gal4	Gal4-Spc110p$^{1–204}$	Gal4-Spc110p$^{1–176}$	Gal4-Spc110p*	
lexA	0.1	0.3	0.3	0.4	
lexA-Spc97p	0.3	201	199	49	
lexA-Spc97–14^{ts}p	0.2	62	n.d.	n.d.	
lexA-Spc97–20^{ts}p	0.2	0.8	n.d.	n.d.	
lexA-Spc98p	0.5	245	222	76	
lexA-Tub4p	2.4	2.5	2.4	n.d.	empty plasmid
lexA-Xgam	0.4	0.5	n.d.	n.d.	
lexA-Tub4p	2.6	2.8	2.3	n.d.	Spc97p
lexA-Tub4p	2.9	12.3	13.9	n.d.	Spc98p
lexA-Tub4p	3.1	162	167	n.d.	Spc97p and Spc98p
lexA-Xgam	0	0.6	n.d.	n.d.	Spc97p and Spc98p
lexA-Tub4–1^{ts}p	0.9	0.3	0.3	n.d.	Spc97p and Spc98p
lexA	0.2	0.1	0.4	n.d.	Spc97p and Spc98p

Cells were grown to early log phase on selective medium containing 2% raffinose. After addition of galactose to 2%, growth was continued for 6 h. β-galactosidase units were measured as described (Ausubel *et al.*, 1988). Mean values of at least three transformants are shown. The range of error is below 10%. Plasmids are listed in Table III. Empty plasmids were used as controls. Co-expression of Spc97p and/or Spc98p together with the Gal4 and lexA fusions occurred from a *CEN-TRP1* plasmid with one or both genes each behind the *Gal1* promoter.

often detected a band migrating approximately with the double molecular weight of ProA–Spc110p. Due to the coiled-coil structure of Spc110p, this band may represent its dimer. Indeed, when isolated SPBs were subjected to non-reductive SDS–PAGE, most of Spc110p migrated with an apparent molecular weight of ~230 kDa. Due to phosphorylation of Spc110p (Friedman *et al.*, 1996) this band had a diffuse appearance. Upon dephosphorylation it shifted to a single, sharp band (Figure 5C). This indicates that oxidation of cysteines leads to a covalent connection between two molecules of Spc110p. To further characterize the interaction of Spc110p with itself, we used two-hybrid constructs which consisted of the N- and the C-terminal domains as well as the coiled-coil region of Spc110p. In this assay, the C-terminus of Spc110p did interact with itself (Figure 5D). In conclusion, Spc110p shows interaction with itself and can be isolated in complex with Spc42p, Cmd1p and a 35 kDa protein.

Discussion

The yeast γ-tubulin complex binds to the N-terminal domain of Spc110p

MTOCs contain defined sites at which microtubules form from their tubulin subunits, a process known as microtubule nucleation (reviewed by Kalt and Schliwa, 1993; Raff, 1996; Pereira and Schiebel, 1997). Numerous genetic and biochemical experiments suggest that γ-tubulin is a universal component of such nucleation sites (Oakley *et al.*, 1990; Horio *et al.*, 1991; Joshi *et al.*, 1992; Moritz *et al.*, 1995b; Sobel and Snyder, 1995; Spang *et al.*, 1996a; Zheng *et al.*, 1995). Based on these results Oakley (1992) proposed that a ring of 13 γ-tubulin molecules in the MTOC interacts directly with tubulin. This relatively simple model was then complicated by the finding that γ-tubulin is part of larger complexes which seem to be present in the cytoplasm as well as at the MTOC (Stearns and Kirschner, 1994; Zheng *et al.*, 1995; Moudjou *et al.*, 1996). This raised questions about the nature and functions of the proteins in the γ-tubulin complexes, the binding of γ-tubulin complexes to a MTOC, the regulation of its activity, and whether the cytoplasmic complexes are identical in composition and structure compared with the complex at the MTOC.

Table II. Synthetic lethality between *spc110-2* and temperature-sensitive alleles of *SPC97*, *SPC98* and *TUB4*

Genetic background	Growth at 23°C	Growth at 30°C
spc110-2	good	good
spc97-14	good	good
spc97-20	good	good
spc110-2/spc97-14	good	reduced
spc110-2/spc97-20	strongly reduced	no
spc98-1	good	good
spc98-2	good	good
spc98-4	good	good
spc98-5	good	good
spc110-2/spc98-1	strongly reduced	no
spc110-2/spc98-2	strongly reduced	no
spc110-2/spc98-4	good	reduced
spc110-2/spc98-5	strongly reduced	no
tub4-1	good	good
spc110-2/tub4-1	good	reduced

Synthetic-lethality was assayed as described in Materials and methods. Temperature-sensitive alleles of *SPC110*, *SPC98*, *SPC97* and *TUB4* have been described previously (Geissler *et al.*, 1996; Kilmartin and Goh, 1996; Spang *et al.*, 1996a; Knop *et al.*, 1997).

In this and previous studies we addressed some of these questions using yeast *S.cerevisiae* as a model system. In *S.cerevisiae* the γ-tubulin Tub4p forms a 6S complex containing the SPB components Spc98p and Spc97p (Geissler *et al.*, 1996; Knop *et al.*, 1997). The affinity purification of this complex identified Tub4p, Spc98p and Spc97p as the only components: no other known SPB component (calmodulin, Kar1p, Spc42p or Spc110p) nor α- or β-tubulin were identified as subunits. Genetic analysis with functional 3HA-tagged Tub4p, and with 3MYC- and 3HA-tagged Spc98p and Spc97p revealed one molecule of Spc98p and Spc97p (this study), but two or more molecules of Tub4p in the complex (Knop *et al.*, 1997). The isolated Tub4p complex (6S) is smaller in size compared with the *X.laevis* γ-tubulin complex (25S) and

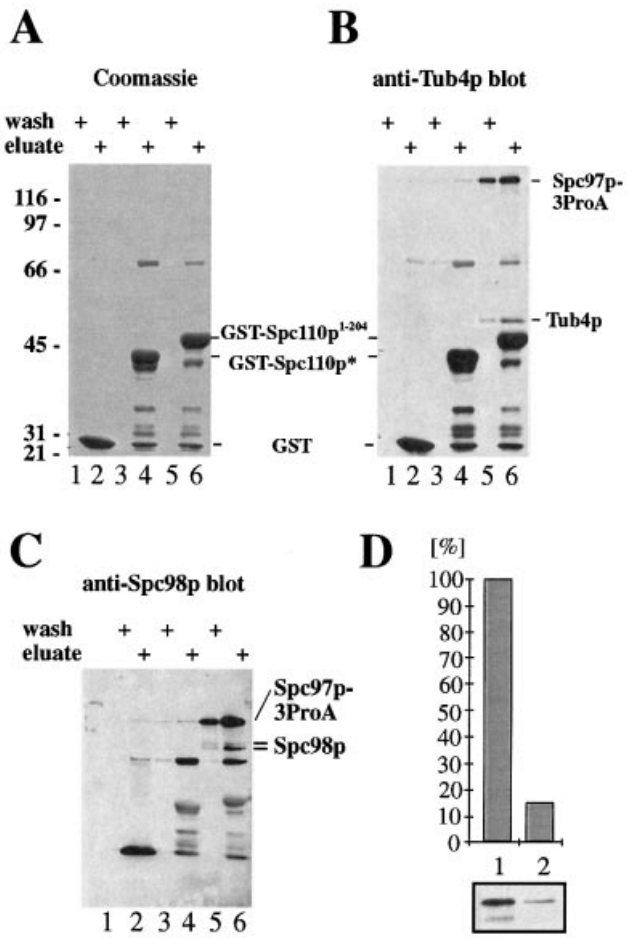

Fig. 4. Biochemical interaction of Spc110p$^{1–204}$ with the γ-tubulin complex. Cells of strain YMK47 (Spc97p-3ProA) were lysed with glass beads (see Materials and methods). The cleared extracts were applied to glutathione–Sepharose columns, on which the indicated fusion proteins were immobilized. After washing the columns with buffer ('wash' denotes the last 500 µl of the wash fraction), bound proteins were eluted with HEPES-G100 containing 10 mM glutathione (eluate). (**A**) Coomassie staining of the wash (odd numbers) and eluate fractions (even numbers). Lanes 1 and 2: GST; lanes 3 and 4: GST-Spc110p*; lanes 5 and 6: GST-Spc110p$^{1–204}$. The bands below the GST-Spc110p* and GST-Spc110p$^{1–204}$ represent degraded fusion proteins. (**B**) Immunodetection of Tub4p in the wash and eluate fractions. The antibody specific for Tub4p was raised against a GST–Tub4p fusion protein and therefore also recognizes the GST–Spc110p fusions on the blot. The protein of ~70 kDa is most probably Hsp70 from *E.coli* which copurifies with many fusion proteins. (**C**) Immunodetection of Spc98p in the wash and eluate fractions. The antibody specific for Spc98p shows weak interaction with GST. (**D**) Binding of GST-Spc110p$^{1–204}$ to purified Tub4p complex bound to latex microspheres. GST-Spc110p$^{1–204}$ was incubated with microspheres with or without Tub4p complex. Unbound GST-Spc110p$^{1–204}$ was removed by centrifugation of the microspheres through a glycerol cushion. The microspheres of Tub4p complex (lane 1) and control beads (lane 2) were analysed for bound GST-Spc110p$^{1–204}$ by immunoblotting. Immunoblots were quantified by densitometry.

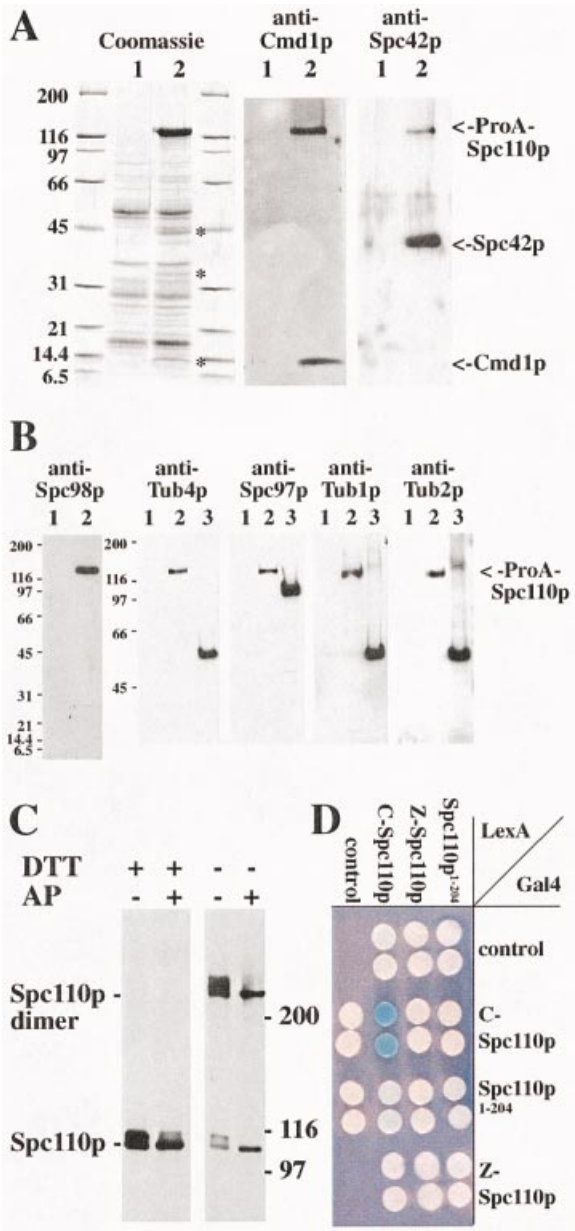

Fig. 5. (**A**) and (**B**) Composition of the Spc110p complex. ProA–Spc110p complexes were purified from cells of strain ESM172 (lane 2; ProA–Spc110p) as described in Materials and methods. As a control, identical incubations were performed with *SPC110* cells (lane 1). Aliquots of these samples were subjected to SDS–PAGE (8–18% gradient gels or 8% gels). Coomassie blue staining of the gel or immunoblots were performed. Antibodies are as indicated. In some cases isolated SPBs were used as a sensitivity control for the immunoblots (lane 3). (**C**) Spc110p interacts with itself. Isolated SPBs were incubated with or without alkaline phosphatase (AP) as indicated and subjected to 5–12% reductive (addition of DTT to the sample buffer) or non-reductive (no DTT) SDS–PAGE gradient gel electrophoresis. Spc110p was detected using specific antibodies. (**D**) The C-terminus of Spc110p does interact with itself. LexA and Gal4 fusions of the indicated subdomains of Spc110p were tested in the two-hybrid system in all possible combinations.

contains only three proteins, while the *X.laevis* complex consists of seven proteins. These proteins include α-, β- and γ-tubulin and proteins with apparent molecular weights of 195, 133, 109 and 75 kDa (Zheng *et al.*, 1995). Whether any of the latter four proteins are homologues of Spc98p or Spc97p is unclear, since their corresponding cDNA has not been published. Why are the two γ-tubulin complexes different in size and composition? The *X.laevis* γ-tubulin complex was purified from frog eggs, containing many proteins stored in the cytoplasm. It could well be that some of the proteins in the *X.laevis* γ-tubulin complex have special storage-specific functions and are not involved in microtubule nucleation. Alternatively, microtubule nucleation in yeast and some other organisms may require

less components, due to more simple microtubule nucleation sites. This hypothesis is supported by the finding that the γ-tubulin complex of *A.nidulans* appears similar in size to the Tub4p complex (Akashi *et al.*, 1997). Finally, we may not have isolated the entire Tub4p complex, but only the most stable core. We are testing the functionality of the isolated Tub4p complex by measuring its microtubule nucleation activity.

γ-Tubulin complexes should have at least two activities. First, they should nucleate microtubules by the binding of one of the subunits of the γ-tubulin complex, most likely γ-tubulin itself, to tubulin. Second, one or more subunits of γ-tubulin complexes must interact with a putative docking protein in MTOCs. Since in many organisms and cell types microtubule nucleation is restricted to MTOCs, binding of cytoplasmic γ-tubulin complexes to docking sites may be one factor that activates the complex. In addition, the number of nucleation sites, which seems to increase in mammalian cells at the onset of mitosis (Kuriyama and Borisy, 1981), may be regulated by the number of active docking sites. This makes the identification of docking proteins an important task.

For the Tub4p complex, we have shown that Spc98p and Spc97p interact in the two-hybrid system with the N-terminal domain of the SPB component Spc110p, while Tub4p had no such activity. Instead, Tub4p interacted with Spc110p$^{1–204}$ only after co-overexpression of *SPC98* and *SPC97*, suggesting that Spc98p and Spc97p, in complex with Tub4p, mediate the interaction. Confirming these results, binding of the Tub4p complex (either from total yeast lysate or purified from yeast extracts) was demonstrated using recombinant GST-Spc110p$^{1–204}$. Taken together, our biochemical and genetic results strongly suggest that Spc98p and Spc97p of the Tub4p complex bind to the N-terminal domain of Spc110p. Due to the allele specificity of the genetic interactions of *spc110(ts)* with *spc98(ts)* or *spc97(ts)* and the binding of GST-Spc110p$^{1–204}$ to purified Tub4p complex, we believe that Spc110p interacts directly with the Tub4p complex, although we can not exclude supporting factors *in vivo*. Spc110p is only associated with the inner plaque, but the Tub4p complex is associated with outer and inner plaques (Rout and Kilmartin, 1990; Spang *et al.*, 1996a,b; Knop *et al.*, 1997). Therefore a not yet identified SPB protein has to function as the docking site for the Tub4p complex at the outer plaque.

A molecular model for a microtubule nucleation site

We purified Spc110p in association with Spc42p, calmodulin and a 35 kDa protein. The fact that no other SPB components such as Spc98p, Spc97p, Tub4p or Kar1p were detectable in the ProA–Spc110p preparation argues that we isolated a defined subcomplex of SPBs and not just randomly fragmented SPBs pieces. This conclusion is further supported by the finding that an extracted Spc110p complex migrated as a single band of 550 kDa on native polyacrylamide gels and with an isoelectric point of 4.2 as determined by isoelectric focusing (E.Schiebel, unpublished). The co-purification of the four proteins indicates that they are in physical contact in the SPB. This is not surprising for Spc110p and calmodulin, since it has been shown that Spc110p has a Ca^{2+}-independent

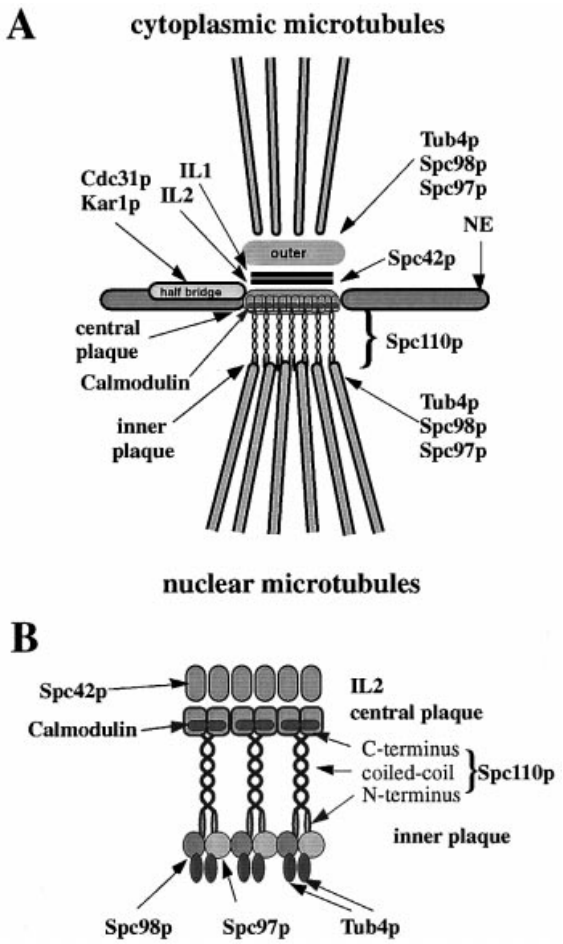

Fig. 6. Model for the attachment of microtubules to the inner plaque of the SPB. (**A**) Localization of known components of the SPB: calmodulin (Spang *et al.*, 1996b; Sundberg *et al.*, 1996), Cdc31p (Spang *et al.*, 1993), Kar1p (Spang *et al.*, 1995), Spc42p (Donaldson and Kilmartin, 1996; Bullitt *et al.*, 1997), Spc98p and Spc110p (Rout and Kilmartin, 1990), Tub4p (Spang *et al.*, 1996a) and Spc97p (Knop *et al.*, 1997). IL1, intermediate layer 1; IL2, intermediate layer 2; NE, nuclear envelope. Nomenclature of the substructures as described by Bullitt *et al.* (1997). (**B**) Schematic drawing of a model for the inner plaque summarizing the results described in this paper.

calmodulin-binding site at its C-terminus (Geiser *et al.*, 1993; Stirling *et al.*, 1994; Spang *et al.*, 1996b). Spc42p is an essential coiled-coil protein that interacts with itself and forms a layer above the central plaque of the SPB (Donaldson and Kilmartin, 1996; Bullitt *et al.*, 1997) (Figure 6A and B). Since the calmodulin-binding site and therefore the C-terminus of Spc110p are close to the inner plaque (Spang *et al.*, 1996b; Sundberg *et al.*, 1996), we suggest that Spc110p is embedded in this layer via an interaction of the C-terminal domain of Spc110p with Spc42p. Initial attempts to show two-hybrid interaction of subdomains of Spc42p and Spc110p have failed so far (M.Knop, unpublished). Therefore, it is unclear whether this interaction is direct or mediated for example through the 35 kDa protein. It is less likely that calmodulin is involved in this interaction, since it is not associated with the SPB when the calmodulin-binding site of Spc110p is deleted (Stirling *et al.*, 1994; Spang *et al.*, 1996b). Remarkably, this deletion had no effect on cell-growth or the structure of the SPB (Geiser *et al.*, 1993; Spang *et al.*, 1996b).

The long coiled-coil domain of Spc110p bridges the distance between the central and the inner plaque. This is suggested from elegant experiments by Kilmartin *et al.* (1993) who showed that truncations within the coiled-coil domain of Spc110p reduced the distance between the inner and central plaque. Our results are consistent with a parallel homodimeric structure of Spc110p (Figure 5C and D). This is further supported by the homology of the coiled-coil domain of Spc110p with tropomyosin (Peitsch, 1996), which forms a parallel two-stranded homodimer (Whitby *et al.*, 1992). Spc98p and Spc97p of the Tub4p complex bind to the N-terminal head domains of this Spc110p dimer which face the nucleoplasm (Spang *et al.*, 1996b). The binding of Spc98p and Spc97p to the N-terminus of Spc110p may determine the topology of the Tub4p complex such that Tub4p is directed away from the SPB and it may further activate the complex. Considering this possibility, the number of Spc110p molecules within the SPB could determine the number of Tub4p complexes at the SPB and therefore the number of microtubules associated with the inner plaque. It is unknown how many Tub4p complexes are required for the nucleation of one microtubule. However, we assume that Tub4p complexes bound to Spc110p may form multimers. Such multimeric structures could form the bell-shaped cap that seals the ends of yeast microtubules that are associated with the inner plaque (Byers *et al.*, 1978). Interestingly, a protein immunologically related to Spc110p has been identified in human centrosomes (A.M.Tassin, unpublished), raising the possibility that it is not only microtubule nucleation by γ-tubulin that is a phylogenetically conserved process, but also binding of γ-tubulin complexes to MTOCs.

Materials and methods

General methods

Yeast strains are listed in Table III. Standard yeast techniques were used to manipulate strains (Guthrie and Fink, 1991). For biochemical purposes, yeast cells were grown on YPAD medium (yeast extract, bacto peptone, adenine and dextrose) to a density of 2×10^7 cells per ml. Cells were either used fresh or frozen in liquid nitrogen and kept at –80°C. *E.coli* strains were transformed by electroporation (Dower *et al.*, 1988). Recombinant DNA methodology was performed as published (Sambrook *et al.*, 1989). DNA sequences of PCR products were determined by PCR sequencing using the reagents and machines of Perkin Elmer.

Plasmids

Plasmids are listed in Table III. Pieces of the *SPC110* open reading frame were amplified by PCR using appropriate primers and then cloned into the plasmids pEG202, pACTII, p423-*Gal1* and pGEX-5X-1 (as indicated in Table III). The point-mutation within *SPC110** leading to the Y15C amino acid exchange was introduced accidentally by PCR (plasmid pSM72, subcloned into plasmid pSM466). Construction of gene fusions: the sequences of ProteinA or 3MYC were amplified by PCR with primers containing flanking *Not*I-sites. Resulting PCR products were digested with *Not*I and then cloned into *Not*I-sites that have been introduced into *SPC98*, *SPC97* or *SPC110* by recombinant PCR. Upon sequencing, the functionality of the resulting gene fusions was tested by their ability to complement the deletion of the wild type gene.

Affinity purification of Spc97p–3ProA- and ProA–Spc98p-containing complexes

Approximately 4 g of yeast cells were lysed with glass beads in lysis buffer (L-buffer; 50 mM Tris–HCl pH 7.6, 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 5% glycerol), containing protease inhibitors [PMSF (1 mM), benzamidine–HCl (350 μg/ml), antipain (6 μg/ml), leupeptin (4.3 μg/ml), aprotinin (4.5 μg/ml), bovine trypsin inhibitor (5 μg/ml), pepstatin A (5 μg/ml) and chymostatin (6 μg/ml)]. After cell breakage 1% Triton X-100 was added. The lysate was incubated on ice for 45 min and then centrifuged (15 min at 12 000 r.p.m. in a SS34 rotor). Purification of the fusion proteins over IgG–Sepharose columns -(Pharmacia) (0.3 ml bed-volume) was essentially done as described (Grandi *et al.*, 1993). The column was washed with the following buffers: L-buffer containing 1% Triton X-100 (20 ml); TBS containing 0.1% Tween 20 (10 ml); 5 mM NH_4OAc pH 5.5 (3 ml) and eluted with 0.5 M HOAc pH 3.4 (1.5 ml). The eluate was dried in a speed vac concentrator.

Pulse-labeling of yeast cells was done as described (Zachariae *et al.*, 1997). Cells were lysed with glass beads in L-buffer containing a 10-fold excess of cold cell lysate of strain CB018 and 1% Triton X-100. Immunoprecipitation was done using anti-HA antibodies covalently coupled to protein G–Sepharose (Sigma). The immunoprecipitates were washed twice with L-buffer with Triton X-100, with 50 mM Tris–HCl pH 7.5, 0.3 M NaCl, with 50 mM Tris–HCl pH 7.5, 0.5 M KCl, with L-buffer containing 0.1 mg/ml of HA-peptide (GYPYDVPDYA) and finally with L-buffer. The bound proteins were separated by SDS–PAGE chromatography, the gel was incubated with Autofluor (National Diagnostics), dried and exposed to a X-ray film.

Affinity purification of ProA–Spc110p complex

Cells (50 g) of a *ProA-SPC110* strain were lysed with glass beads in L-buffer as described above. The insoluble material, containing >95% of all ProA–Spc110p and <1% of the components of the Tub4p complex, was harvested (15 min at 12 000 r.p.m. in a SS34 rotor) and incubated in 130 ml high-salt L-buffer (containing 1 M NaCl, 1% Triton X-100 and protease inhibitors as above) while stirring vigorously for 45 min. The extract was diluted to 0.35 M NaCl followed by centrifugation (15 min at 12 000 r.p.m. in a GSA rotor). The supernatant containing 50–60% of total ProA–Spc110p was applied to a IgG–Sepharose column (1.3 ml bed volume). The column was washed with the following buffers: L-buffer containing 0.35 M NaCl, 1% Triton X-100 (20 ml); L-buffer containing 0.5 M NaCl, 1% Triton X-100 (5 ml); L-buffer containing 0.5 M KCl instead of NaCl, 1% Triton X-100 (5 ml), TBS with 0.1% Tween 20 (20 ml), 5 mM ammonium acetate (NH_4OAc) pH 6.5 (5 ml), 5 mM NH_4OAc pH 5.5 (5 ml) and 5 mM NH_4OAc pH 5.0 (5 ml). Proteins were eluted with 6.5 ml 0.5 M acetic acid pH 3.4. Finally, the eluate was dried.

Isolation of SPBs and alkaline phosphatase treatment

SPBs were isolated from a proteinase-deficient strain CB018 according to Rout and Kilmartin (1990). For alkaline phosphatase treatment, the SPBs were TCA precipitated, the precipitates were washed with 70% ethanol (–20°C), rinsed with 1 M Tris–HCl pH 8 (0°C) and solubilized in 1% SDS (5 min 95°C). Dephosphorylation with alkaline phosphatase (Boehringer Mannheim) was carried out in AP-buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.4% SDS) for 45 min at 30°C.

Stoichiometry of the Tub4p complex

Crude cell lysates corresponding to 5×10^8 cells of strains expressing either *SPC97* and *SPC97-3HA*, *SPC97-3MYC* and *SPC97-3HA* or *Spc97* and *SPC97-3MYC* were prepared (Knop *et al.*, 1997). Immunoprecipitations were carried out with 12CA5 monoclonal antibody directed against the HA epitope and with polyclonal goat anti-Spc98p antibodies and the precipitates were analyzed by immunoblotting. An identical experimental setup was used to analyze the stoichiometry of Spc98p.

Synthetic lethality

spc110-2 cells (YMK83) were transformed with *CEN-URA3* plasmids containing either *SPC97* (pMK10), *SPC98* (pSM296) or *TUB4* (pSM223). Thereafter the chromosomal copy of the respective gene was disrupted using an appropriate disruption cassette (Δ*spc97::HIS3*, pMK6; Δ*spc98::HIS3*, pSM294; Δ*tub4::HIS3*, pSM219). Growth of these cells was now dependent on the wild type gene on the *URA3*-based plasmid. To test, whether temperature-sensitive alleles were synthetic-lethal, these strains were transformed with *CEN-LEU2* plasmids harbouring the temperature-sensitive alleles (see Table III). For controls, *CEN-TRP1* plasmids with or without *SPC110* were co-transformed. Several transformants were assayed on 5′-FOA plates at 23°C and 30°C for their ability to lose the wild-type gene on the *URA3*-based plasmid.

Two-hybrid assays

Gene fusions with the DNA-binding domain of *lexA* were made using plasmid pEG202 (Gyuris *et al.*, 1993). Plasmid pACTII was chosen as activation domain (Gal4p) vector (Durfee *et al.*, 1993). Strain SGY37

Table III. Yeast strains and plasmids

Name	Genotype: construction	Source or reference
Yeast strains		
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH500	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YMK18	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK26	Knop *et al.* (1997)
YMK22	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK29	Knop *et al.* (1997)
YMK47	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK38	this study
YMK83	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc110-2*	this study
YMK90	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc98::HIS3* pSM291 pSM375	this study
YMK91	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc98::HIS3* pSM346 pSPC98	this study
YMK92	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc98::HIS3* pSM346 pSM375	this study
YMK93	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc97::HIS3* pMK28 pSM376	this study
YMK94	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc97::HIS3* pMK10 pMK31	this study
YMK95	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1Δspc97::HIS3* pMK31 pSM376	this study
YMK103	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc110-2 Δtub4::HIS3* pSM223	this study
YMK104	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc110-2 Δspc98::HIS3* pSM296	this study
YMK105	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc110-2 Δspc97::HIS3* pMK10	this study
ESM172	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc110:HIS3* pSM192	this study
ESM282	*MAT*α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pSM340	this study
ESM387-3	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 leu2Δ1:Gal1-TUB4:LEU2 trp1Δ63:Gal1-SPC97-3HA:TRP1 ura3-52:Gal1-SPC98:URA3*	this study
SGY37	*MAT***a** *ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2*	Geissler *et al.* (1996)
CB018	*MAT***a** *ura3-1 his3-11,15 leu2-3,112 ade2-1^{oc} trp1-1 can1-100 Δpep4::HIS3 Δprb1::hisG Δprc1::hisG*	from R. Fuller, for Ref. see Graham and Emr (1991)
Plasmids		
pACTII	2 μm, *LEU2*-based vector carrying the *GAL4* activator domain	Durfee *et al.* (1993)
pEG202	2 μm, *HIS3*-based vector carrying the *lexA* DNA-binding domain	Gyuris *et al.* (1993)
pMK6	pBluescript SK$^+$ containing Δ*spc97::HIS3*	Knop *et al.* (1997)
pSM294	pBluescript SK$^+$ containing Δ*spc98::HIS3*	Geissler *et al.* (1996)
pSM219	pBluescript SK$^+$ containing Δ*tub4::HIS3*	Spang *et al.* (1996)
pMK10	pRS316 containing *SPC97*	Knop *et al.* (1997)
pMK15	pACTII containing *SPC97*	Knop *et al.* (1997)
pMK16	pEG202 containing *SPC97*	Knop *et al.* (1997)
pMK26	pRS414 containing *SPC97*	Knop *et al.* (1997)
pMK28	pRS414 containing *SPC97* (with *Not*I restriction site before the STOP codon)	Knop *et al.* (1997)
pMK29	pRS414 containing *SPC97-3HA*	Knop *et al.* (1997)
pMK31	pRS414 containing *SPC97-3MYC*	this study
pMK38	pRS414 containing *SPC97-3ProA*	this study
pMK51	p414-*Gal1* containing *SPC97*	this study
pMK103	pEG202 containing *spc97-14*	Knop *et al.* (1997)
pMK104	pEG202 containing *spc97-20*	Knop *et al.* (1997)
pMK151	pACTII containing *SPC110*$^{1-204}$ (codons for amino acids 1–204)	this study
pMK150	pACTII containing *C-SPC110* (codons for amino acids 823–STOP)	this study
pMK155	p414-*Gal1* containing *SPC97* with *Gal1-SPC98* in reverse orientation	this study
pMK171	pGEX-5X-1 with *SPC110*$^{1-204}$	this study
pMK173	pACTII-*SPC110*$^{1-176}$ (codons for amino acids 1–176)	this study
p*spc97-14*	pRS315 containing *spc97-14*	this study
p*spc97-20*	pRS315 containing *spc97-20*	this study
pSG26	pACTII containing *SPC98*	Geissler *et al.* (1996)
pSG11	pEG202 containing *SPC110*$^{1-204}$	this study
pSG12	pEG202 containing *Z-SPC110* (codons for amino acids 146–846)	this study
pSG13	pEG202 containing *C-SPC110* (codons for amino acids 823–STOP)	this study
pSG28	pACTII containing *Z-SPC110* (codons for amino acids 146–846)	Geissler *et al.* (1996)
pSG35	pEG202 containing γ-tubulin from *X.laevis* (Xgam)	Geissler *et al.* (1996)
pSG40	pEG202 containing *tub4-1*	Geissler *et al.* (1996)
pSG56	pEG202 containing *SPC98*	Geissler *et al.* (1996)
pSM72	pGEX-3x with *SPC110** (codons for amino acids 3–176 of Spc110p with Y15C)	Spang *et al.* (1996a)
pSM192	pRS414 containing *ProA-SPC110*	this study
pSM204	pRS315 containing *tub4-1*	Spang *et al.* (1996b)
pSM223	pRS316 containing *TUB4*	Spang *et al.* (1996b)
pSM291	pRS315 containing *SPC98*	Geissler *et al.* (1996)
pSM296	pRS316 containing *SPC98*	Geissler *et al.* (1996)
pSM340	pRS315 containing *ProA-SPC98*	this study
pSM346	pRS315 containing *3MYC-Spc98*	this study
pSM375	pRS314 containing *3HA-SPC98*	this study
pSM376	pRS315 containing *SPC97-3HA*	this study
pSM438	p414-*Gal1* containing *SPC98*	this study
pSM459	p423-*Gal1* containing *SPC110*$^{1-204}$	this study
pSM461	p423-*Gal1* containing *SPC110*$^{1-176}$	this study
pSM466	pACTII containing *SPC110** (codons for amino acids 3–176 of Spc110p with Y15C)	this study

Table III. *Continued*

Name	Genotype: construction	Source or reference
pSPC98	pRS314 containing *SPC98*	Knop *et al.* (1997)
p*spc98-1*	pRS315 containing *spc98-1*	Geissler *et al.* (1996)
p*spc98-2*	pRS315 containing *spc98-2*	this study
p*spc98-4*	pRS315 containing *spc98-4*	this study
p*spc98-5*	pRS315 containing *spc98-5*	this study
pCM102	pRS414 containing *SPC110*	this study
pRS314	*CEN6*, *TRP1*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS414	pRS314 with inversed multi cloning site	Sikorski and Hieter (1989)
pRS315	*CEN6*, *LEU2*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS316	*CEN6*, *URA3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS423	2 μm, *HIS3*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pRS425	2 μm, *LEU2*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pRS426	2 μm, *URA3*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
p414-*Gal1*	pRS414 containing the *Gal1*-promotor	Mumberg *et al.* (1995)
p423-*Gal1*	pRS423 containing the *Gal1*-promotor	Mumberg *et al.* (1995)
pGEX-5X-1	*E.coli* expression vector containing GST under control of the lacZ promotor	Pharmacia
pGEX-3X	*E.coli* expression vector containing GST under control of the lacZ promotor	Pharmacia

was simultaneously transformed with the indicated plasmids and two-hybrid interaction was tested on X-gal indicator plates (Gyuris *et al.*, 1993) containing 2% raffinose, 2% galactose and lacking histidine and leucine (experiments shown in Figures 3A and 6B) and eventually tryptophan (experiment in Figure 3B). β-Galactosidase assays of permeabilized cells and calculation of the specific activities were done as described (Ausubel *et al.*, 1988).

Binding of the Tub4p complex to recombinant GST-Spc110p$^{1–204}$

Recombinant GST fusions were expressed in *E.coli* strain DH5α for 3 h at 37°C and bound to glutathione–Sepharose (300 μl bed volume on disposable plastic columns) according to the manufacturer's recommendation (Pharmacia). The beads were washed with PBT (PBS with 0.1% Triton X-100), PBS and HEPES-G100 buffer (50 mM Na-HEPES pH 8.0, 100 mM NaCl, 5% glycerol, 2 mM EGTA, 1 mM $MgCl_2$, 0.1 mM GTP). Plasmids were pGEX-5X-1 (GST), pSM72 (GST-Spc110*, amino acids 3–176, Y15C) and pMK171 (GST-Spc110p$^{1–204}$). An extract of cells from strain YMK47 (*SPC97-3ProA*) in HEPES-G100 containing proteinase inhibitors was made using glass beads. Cell debris was removed (15 min 12 000 r.p.m. in a SS34 rotor) and lysate corresponding to 2×10^9 cells in a volume of 10 ml was applied per column to the bound GST fusion proteins. After washing with HEPES-G100 (15 ml), glutathione–Sepharose bound proteins were eluted with HEPES-G100 containing 10 mM glutathione.

Non-specific rabbit IgGs (Dianova) were cross-linked to carboxylated latex microspheres with a diameter of 0.2 μm according to the manufacturer's instruction (Molecular Probes). The beads were kept as a 2% suspension in PBS with 1% BSA. One gram of yeast cells of strains YMK47 (*SPC97-ProA*; Tub4p beads) or YMK18 (*SPC97*; control) were lysed with glass beads in L-buffer with 1% Triton X-100 and inhibitors and the lysates were adjusted to a total volume of 10 ml and 1% Triton X-100. The lysates were centrifuged [two spins at 28 000 r.p.m. for 15 min in a Ti70.2 rotor (Beckman)] and the supernatants were incubated with 50 μl of IgG beads for 12 h. The beads were washed twice with L-buffer with 1% Triton X-100, twice with TBS with 0.1% Tween 20, twice with 5 mM NH_4OAc pH 5.5 and once with HEPES-G100. The beads were resuspended in 3 ml HEPES-G100 containing 1% BSA. The Tub4p beads, but not the control beads, contained Spc97p–ProA, Tub4p and Spc98p as determined by immunoblotting and Coomassie Blue staining (data not shown). Purified GST-Spc110p$^{1–204}$ was mixed with 500 μl beads and layered on top of a 3 ml cushion of HEPES-G100 with 15% glycerol. The beads were spun for 1 h [20 000 r.p.m., SW60 rotor (Beckman)] onto the top of a 0.5 ml 87% glycerol cushion. The beads were collected and adjusted to 500 μl and 1% SDS. The amount of co-fractionating GST-Spc110p$^{1–204}$ was determined by immunoblotting.

Immunological techniques

Antibodies specific for Spc97p were produced against a recombinant, affinity purified 6xHis-Spc97$^{49–415}$ protein expressed from plasmid pSM387 in *E.coli* strain SG13009 (Diagen). The anti-Spc98p (Knop *et al.*, 1997), anti-Tub4p (Spang *et al.*, 1996a), anti-Kar1p (Spang *et al.*, 1995), anti-Spc42p [prepared as described by Donaldson and Kilmartin (1996)], anti-Spc110p and anti-Cmd1p (Spang *et al.*, 1996b) antibodies have been described before.

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