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. In a model, 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 collectivelly 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 substoichiometric amounts (Figure 1C). An identical result for the composition of this complex was obtained by native immunoprecipitation of 3HA-tagged Spc97p from [35S]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-3HA.
An identical experimental setup was used for SPC97 identical blots were made and probed with the indicated antibodies. The immunoprecipitates were solubilized in sample buffer and four et al. 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<sup>1–204</sup>) 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<sup>1–176</sup>) were tested. Mutated versions of Spc97p (Knop et al., 1997) showed weaker (Spc97-14p) or no (Spc97-20p) interaction with Spc110p<sup>1–204</sup> (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<sup>1–204</sup>. This was indeed the case: when we co-overexpressed Spc97p and Spc98p from the strong Gal1-promoter together with Gal4-SPC110<sup>1–204</sup>, we obtained a strong signal in the two-hybrid system with lexA-TUB4 (Figure 3B), but not with lexA-tub4-1<sup>1</sup>α 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<sup>ts</sup>, spc98<sup>ts</sup> or tub4-1<sup>1</sup>α. Synthetic lethality is an indication for a functional relationship of two genes. spc110-2 was synthetically-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<sup>1–204</sup> with the γ-tubulin complex came from overexpression experiments. Strong overexpression of SPC110<sup>1–204</sup> was toxic to cells. This toxicity was increased when SPC110<sup>1–204</sup> 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<sup>1–204</sup>. 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).
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\(^{1-204}\) 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 in wild type cells YPH499 or in cells of strain ESM387-3 (YPH499 containing integrated Gal1-TUB4 Gal1-SPC110 co-overproduced Spc98p and/or Spc97p. The empty plasmid was used as a control (–). These three proteins appear to be specifically associated with ProA–Spc110p, as they marked with an asterisk). These three proteins are not Tub4p, interact directly with the N-terminal domain of Spc110p.

![Fig. 3. Interaction of Spc110p with components of the γ-tubulin complex.](image)

### 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). 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). 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).
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., 1995a; 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.

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; Kilmartin and Goh, 1996; Spang et al., 1996a; Knop et al., 1997). The affinity purification of this complex identified Tub4p, Spc98p and Spc97p as the only components: no other known SPB 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...
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 Spc110p1–204 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-Spc110p1–204. 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-Spc110p1–204 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 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). 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.

[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., 1996) 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.]
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 \( \gamma \)-tubulin that is a phylogenetically conserved process, but also binding of \( \gamma \)-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 \( 2 \times 10^7 \) 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 SPSC110 gene fusions: the sequences of ProteinA or 3MYC were amplified by PCR using appropriate primers and then cloned into plasmid pSM72 (as subcloned into plasmid pSM466). Construction of synthetic Gal1 frame were amplified by PCR using appropriate primers and then cloned into plasmid pEG202 (Gyuris et al., 1997).

**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 \( \mu \)g/ml), antipain (6 \( \mu \)g/ml), leupeptin (4.3 \( \mu \)g/ml), aprotinin (4.5 \( \mu \)g/ml), bovine trypsin inhibitor (5 \( \mu \)g/ml), pepstatin A (5 \( \mu \)g/ml) and chymostatin (6 \( \mu \)g/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 NH4OAc 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.

**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 (NH4OAc) pH 6.5 (5 ml), 5 mM NH4OAc pH 5.5 (5 ml) and 5 mM NH4HAc 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 9.8, 150 mM NaCl, 1 mM MgCl2, 0.1 mM ZnCl2, 0.4% SDS) for 45 min at 30°C.

**Stochiometry of the Tub4p complex**

Crude cell lysates corresponding to \( 5 \times 10^8 \) cells of strains expressing either SPYC97 and SPYC97-3MYC and SPYC97-3HA or Spc97 and SPYC97-3MYC 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 stochiometry of Spc98p.

**Synthetic lethality**

spc110::HIS3, spc110::URA3, spc110::LEU2, spc110::TRP1 plasmids containing either SPYC97 (pMK10), SPYC98 (pSM296) or TUB4 (pSM223). Thereafter the chromosomal copy of the respective gene was disrupted using an appropriate disruption cassette (\( \Delta spc97::HIS3, pMK6; \Delta spc98::HIS3, pSM294; \Delta tub4::HIS3, pSM219 \)). 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype: construction</th>
<th>Source or reference</th>
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<td>YPH499</td>
<td><strong>M</strong>ατα  ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</td>
<td>Sikorski and Hieter (1989)</td>
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<td><strong>M</strong>ατα  ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 pMK26</td>
<td>Knop et al. (1997)</td>
</tr>
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<td>this study</td>
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<td>YMK93</td>
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<td>YMK95</td>
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<td>YMK104</td>
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<td>SGY37</td>
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<td>CB018</td>
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<td>see Graham and Emr (1991)</td>
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</table>

Plasmids

- **pACTII** 2 μm, LEU2-based vector carrying the **GAL4** activator domain
- **pEG202** 2 μm, HIS3-based vector carrying the lexA DNA-binding domain
- **pMK6** pBlueScript SK- containing Asp97::HIS3
- **pSM294** pBlueScript SK- containing Asp98::HIS3
- **pSM219** pBlueScript SK- containing Δub4::HIS3
- **pMK10** pRS31 containing SPC97
- **pMK15** pACTII containing SPC97
- **pMK16** pEG202 containing SPC97
- **pMK26** pRS414 containing SPC97
- **pMK28** pRS414 containing SPC97 (with Nor1 restriction site before the STOP codon)
- **pMK29** pRS414 containing SPC97-3HA
- **pMK31** pRS414 containing SPC97-3MYC
- **pMK38** pRS414 containing SPC97-3ProA
- **pMK51** p414-Gal1 containing SPC97
- **pMK103** pEG202 containing spc97-14
- **pMK104** pEG202 containing spc97-20
- **pMK151** pACTII containing SPC110^1-204 (codons for amino acids 1–204)
- **pMK150** pACTII containing C-SPC110 (codons for amino acids 823–STOP)
- **pMK155** p414-Gal1 containing SPC97 with Gal1-SPC98 in reverse orientation
- **pMK171** pGEX-5x-1 with SPC110^1-204
- **pMK173** pACTII-SPC110^1-176 (codons for amino acids 1–176)
- **pspc97-14** pRS315 containing spc97-14
- **pspc97-20** pRS315 containing spc97-20
- **pspc97-20** pRS315 containing spc97-20
- **pSG26** pACTII containing SPC98
- **pSG11** pEG202 containing SPC110^1-204
- **pSG12** pEG202 containing Z-SPC110 (codons for amino acids 146–846)
- **pSG13** pEG202 containing C-SPC110 (codons for amino acids 823–STOP)
- **pSG28** pACTII containing Z-SPC110 (codons for amino acids 146–846)
- **pSG35** pEG202 containing γ-tubulin from *X.laevia* (Xgam)
- **pSG40** pEG202 containing tub4-1
- **pSG56** pEG202 containing SPC98
- **pSG72** pGEX-3x with SPC110^* (codons for amino acids 3–176 of Spc110p with Y15C)
- **pSM192** pRS414 containing Pro4-SPC110
- **pSM204** pRS15 containing tub4-1
- **pSM223** pRS316 containing TUB4
- **pSM291** pRS15 containing SPC98
- **pSM296** pRS15 containing SPC98
- **pSM340** pRS15 containing Pro4-SPC98
- **pSM346** pRS15 containing 3MYC-Spc98
- **pSM375** pRS15 containing 3HA-SPC98
- **pSM376** pRS15 containing SPC97-3HA
- **pSM438** p414-Gal1 containing SPC98
- **pSM459** p423-Gal1 containing SPC110^1-204
- **pSM461** p423-Gal1 containing SPC110^1-176
- **pSM466** pACTII containing SPC110^* (codons for amino acids 3–176 of Spc110p with Y15C)
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 leuine (experiments shown in Figures 3A and 6B) and eventually trypton (experimen 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**

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 MgCl2, 0.1 mM GTP). Plasmids were pGEX-5X-1 (GST), pSM72 (GST-Spc110p, amino acids 3–176, Y15C) and pMK171 (GST-Spc110p 1–204). A high copy plasmid, p423-1995, anti-Spc42p [prepared as described by Donaldson and Kilmartin (1995)] and pRS414 containing the lacZ promoter.

**Immunological techniques**

Antibodies specific for Spc97p were produced against a recombinant, affinity purified 6His-Spc97p protein expressed from plasmid pSM387 in E. coli strain S13009 (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)] and anti-Spc110p and anti-Cmd1p (Spang et al., 1996b) antibodies have been described before.

**Acknowledgements**

Rabbit-anti-Tub1p and rabbit-anti-Tub2p sera were kind gifts from F.Solomon. We thank A.M.Tassin for communicating results before publication. We gratefully acknowledge Katrin Grein and Elvira Glatt for technical assistance. This work was carried out with support from the BMBF.

**References**


Donaldson,A.D. and Kilmartin,J.V. (1996) Spc42p: A phosphorylated afﬁnity puriﬁed 6His-Spc97p protein expressed from plasmid pSM387 in E. coli strain S13009 (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.

**Table III. Continued**

<table>
<thead>
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<th>Name</th>
<th>Genotype: construction</th>
<th>Source or reference</th>
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<tr>
<td>pSPC98</td>
<td>pRS314 containing SPC98</td>
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<td>pspc98-1</td>
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<td>pspc98-4</td>
<td>pRS315 containing spc98-4</td>
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<td>pspc98-5</td>
<td>pRS315 containing spc98-5</td>
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<td>pCM102</td>
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<td>pRS314</td>
<td>CEN6, TRP1-based yeast–E.coli shuttle vector</td>
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<td>CEN6, LEU2-based yeast–E.coli shuttle vector</td>
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<td>CEN6, URA3-based yeast–E.coli shuttle vector</td>
<td>Christianson et al. (1992)</td>
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<td>pRS423</td>
<td>2 μm, HIS3-based yeast–E.coli shuttle vector</td>
<td>Christianson et al. (1992)</td>
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<td>pRS425</td>
<td>2 μm, LEU2-based yeast–E.coli shuttle vector</td>
<td>Christianson et al. (1992)</td>
</tr>
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<td>pRS426</td>
<td>2 μm, URA3-based yeast–E.coli shuttle vector</td>
<td>Christianson et al. (1992)</td>
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<td>pRS414 containing the Gal1-promotor</td>
<td>Mumberg et al. (1995)</td>
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<td>p423-Gal1</td>
<td>pRS423 containing the Gal1-promotor</td>
<td>Mumberg et al. (1995)</td>
</tr>
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<td>E.coli expression vector containing GST under control of the lacZ promoter</td>
<td>Pharmacia</td>
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<td>pGEX-3X</td>
<td>E.coli expression vector containing GST under control of the lacZ promoter</td>
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