Interaction of the yeast γ-tubulin complex-binding protein Spc72p with Kar1p is essential for microtubule function during karyogamy

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The spindle pole body component Kar1p has a function in nuclear fusion during conjugation, a process known as karyogamy. The molecular role of Kar1p during this process is poorly understood. Here we show that the yeast γ-tubulin complex-binding protein Spc72p interacts directly with the N-terminal domain of Kar1p, thereby targeting the γ-tubulin complex to the half bridge, a substructure of the spindle pole body, where it organizes microtubules. This binding of Spc72p to Kar1p has only a minor role during vegetative growth, whereas it becomes essential for karyogamy in mating cells, explaining the important role of Kar1p in this process. We also show that the localization of Spc72p within the spindle pole body changes throughout the cell cycle and even more strongly in response to mating pheromone. Taken together, these observations suggest that the reallocation of Spc72p within the spindle pole body is the ‘landmark’ event in the pheromone-induced reorganization of the cytoplasmic microtubules.

Keywords: γ-tubulin complex/Kar1p/karyogamy/mating/Spc72p

Introduction

In budding yeast, microtubule organizing functions are provided by the spindle pole body (SPB), a multi-layered structure that is embedded in the nuclear envelope throughout the cell cycle (Byers and Goetsch, 1975). The SPB organizes two main sets of microtubules. The cytoplasmic outer plaque and the nuclear inner plaque are SPB substructures that are associated with the cytoplasmic and nuclear microtubules, respectively. An additional SPB substructure that functions in cytoplasmic microtubule (CM) organization is the half bridge, a one-sided extension of the central plaque layered on top of the nuclear envelope (Byers and Goetsch, 1975). Nuclear and cytoplasmic microtubules are attached to the inner and outer plaque of the SPB throughout the cell cycle (Byers and Goetsch, 1975; illustrated in Figure 8A). In contrast, CMs originate only in G1 of the cell cycle from the SPB half bridge (Byers and Goetsch, 1975). During vegetative growth the nuclear microtubules are involved in SPB separation, spindle formation and chromosome segregation (Jacobs et al., 1988), whereas the CMs function in SPB orientation, spindle positioning and in migration of the nucleus into the bud (Palmer et al., 1992; Carminati and Stearns, 1997; Shaw et al., 1997).

Upon the exposure of yeast cells to mating pheromone the CMs reorganize such that they are now directed from the half bridge of the SPB towards the mating projection (Byers and Goetsch, 1975; Brachat et al., 1998). Following this microtubule reorganization, α and β cells fuse, the two nuclei are moved together along the interdigitating microtubules and finally fuse, a step known as karyogamy (reviewed by Rose, 1996). Genes involved in karyogamy have been identified by genetic screens (Conde and Fink, 1976; Kurihara et al., 1996). Kar1p is particularly interesting since it is found at the half bridge of the SPB (Spang et al., 1995) and functions in both karyogamy (Conde and Fink, 1976; Vallen et al., 1992) and SPB duplication (Rose and Fink, 1987). However, little is known about the molecular role of Kar1p in karyogamy and its possible involvement in the pheromone-induced rearrangement of the CMs at the SPB.

How microtubules form in vivo became clearer with the discovery of γ-tubulin as a universal component of centrosomes and SPBs that is involved in microtubule formation (Oakley and Oakley, 1989; Oakley et al., 1990; Stearns et al., 1991; Stearns and Kirschner, 1994). γ-tubulin is found as part of a large complex (Zheng et al., 1995) containing γ-tubulin, homologues of yeast Spc98p and Spc97p (Rout and Kilmartin, 1990; Geissler et al., 1996; Knop et al., 1997; Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998) and additional proteins, depending on the organism (Zheng et al., 1995; Murphy et al., 1998). In budding yeast the γ-tubulin or Tub4p complex consists of the γ-tubulin, named Tub4p, and the SPB components Spc98p and Spc97p (Rout and Kilmartin, 1990; Geissler et al., 1996; Knop et al., 1997; Knop and Schiebel, 1997, 1998). Like other γ-tubulin complexes (Stearns and Kirschner, 1994; Moudjou et al., 1996), the Tub4p complex assembles in the cytoplasm (Pereira et al., 1998) and is then targeted to the sites of microtubule organization at the SPB via the binding of Spc97p and Spc98p to the N-terminal domain of Spc72p (Knop and Schiebel, 1998) and of Spc110p (Knop and Schiebel, 1997; Nguyen et al., 1998). Spc72p is associated with the cytoplasmic outer plaque, whereas Spc110p is located with the nuclear inner plaque, explaining how the Tub4p complex is bound to the cytoplasmic and nuclear sides of the SPB (Rout and Kilmartin, 1990; Spang et al., 1996; Knop et al., 1997; Wigge et al., 1998). The term γ-tubulin complex-binding protein (GTBP) has been coined for proteins such as Spc72p and Spc110p that target γ-tubulin complexes to distinct cellular localizations (Knop and Schiebel, 1998).
How Spc110p is embedded within the SPB is relatively well understood. Spc110p has been purified in a complex containing yeast calmodulin (Cmd1p), and the SPB components Spc29p and Spc42p (Knop et al., 1997). Spc42p forms a layer within the centre of the SPB (Bullitt et al., 1997) to which the C-terminus of Spc110p is anchored via Spc29p (Elliot et al., 1999). Cmd1p binds to a site in the C-terminus of Spc110p (Geiser et al., 1993; Stirling et al., 1994) which is adjacent to the Spc29p-binding domain and evidence has been obtained that Cmd1p binding to Spc110p may regulate the interaction of Spc110p with Spc29p (Elliot et al., 1999). In contrast, the binding proteins of Spc72p at the SPB have not been identified. However, it is known that the C-terminus of Spc72p is sufficient to target this protein to the SPB (Knop and Schiebel, 1998).

How the CMs of the half bridge are organized in G1 of the cell cycle and in mating cells is largely unknown. Here we provide the first evidence that the Tub4p complex-binding protein Spc72p interacts directly with the N-terminal domain of the half bridge component Kar1p, explaining the function of Kar1p in conjugation. By means of this interaction the Tub4p complex is targeted to the half bridge where it organizes CMs. We also provide evidence that the interaction of Kar1p with Spc72p has only a minor function during vegetative growth, but becomes essential for CM organization during conjugation.

Results

The C-terminus of Spc72p interacts with the N-terminal domain of Kar1p

*KAR1* was identified in a genetic screen for mutants with a defect in karyogamy (Conde and Fink, 1976) and although Kar1p has been localized to the SPB half bridge (Spang et al., 1995), its function during karyogamy has remained elusive. Using the yeast two-hybrid system, we found that amino acids 116–236 of Kar1p interact with the C-terminus of the Tub4p complex-binding protein Spc72p (Spc72p231–622; Figure 1A). This interaction was found to be specific, because it was drastically affected by the kar1-1 and kar1-Δ15 mutations (Figure 1B, lane 3), and no interaction with GST (lane 1) was detectable. In addition, Kar1p bound directly to Spc72p231–622 in vitro when both purified proteins were incubated (data not shown).

To verify the interaction between Kar1p and Spc72p, we expressed *KAR1*, kar1-1, kar1-Δ15 and *SPC72* in wild-type and kar1-Δ15 cells with the control plasmid pRS426. This deficiency of kar1-1 cells was in part suppressed by *SPC72* on the multi-copy plasmid pRS426 (bar 3). Taken together, these data show that Kar1p interacts directly with Spc72p.

In vivo binding of Spc72p to the SPB half bridge is dependent on the N-terminal domain of Kar1p

Spc72p was shown to be a component of the outer plaque (Knop and Schiebel, 1998; Wigge et al., 1998) and evidence has been obtained that it is also associated with the half bridge (Souèes and Adams, 1998; Adams and Kilmartin, 1999). To test whether the localization of Spc72p with the half bridge depends on Kar1p in vivo, we made use of the kar1-1Δ15 mutation (Vallen et al., 1992), which lacks the Spc72p-binding domain of Kar1p (illustrated in Figure 3A). Cells of kar1-1Δ15 do not show an obvious phenotype during vegetative growth, however, they are deficient in karyogamy during conjugation, caused by a CM organization defect (Vallen et al., 1992). Spc72p was found to be associated with the SPB in wild-type cells (Figure 2B, 1 and 2) and in cells of kar1-1Δ15 (Figure 2B, 5–7, red and white arrows) during vegetative growth throughout the cell cycle. However, upon the addition of α-factor (Figure 2B, 5–7, kar1-1Δ15, black arrow in 5; note that α-factor arrested and vegetatively growing cells were mixed to allow a direct comparison of the signals) Spc72p was no longer detectable at the SPB of kar1-1Δ15 cells, whereas it was still associated with the SPB of pheromone-treated wild-type cells (Figure 2B, 3 and 4, white arrows in 4). We established by immunoblotting with anti-N-Spc72p antibodies that the cellular levels of Spc72p were similar in *KAR1* and kar1-1Δ15 cells with or without α-factor (data not shown).

The Kar1p-dependent association of Spc72p with the SPB in mating cells was confirmed in zygotes. Two *SPC72*-GFP cells of opposite mating type were incubated and the Spc72p-GFP signals were followed over time. The cell bodies fused and before karyogamy two Spc72p-GFP signals were observed, each associated with the SPB of the two nuclei (Figure 2A, upper). Upon longer incubation the two nuclei and the SPB fused (Figure 2A, lower right, black arrow) the two nuclei did not fuse, even after a prolonged incubation, due to the karyogamy defect caused by the kar1-1Δ15 mutation (Vallen et al., 1992). Importantly, only the SPB of the wild-type cell carried a partial suppression of the karyogamy defect of kar1-1 cells through high gene dosage of *SPC72* (Figure 1E). The ratio of zygotes to diploids was low when wild-type cells were crossed with each other (bar 2) and this value was not affected by *SPC72* on plasmid pRS426 (bar 1). This reflects the fact that during mating of wild-type cells the two cell bodies and the nuclei fuse to give a zygote which then multiplies during the course of the experiment and thereby gives rise to diploid cells which again divide. In contrast, kar1-1 cells are karyogamy deficient when crossed to wild-type and the progenies of such a zygote are haploid containing one of the two parental nuclei (reviewed by Rose, 1996). Therefore, the ratio of zygotes to diploids is high (bar 4, kar1-1 cells with the control plasmid pRS426).
Fig. 1. The C-terminal domain of Spc72p interacts with the N-terminus of Kar1p. (A) Kar1p and Spc72p interact in the yeast two-hybrid system. Plasmid pACT2 (no insert) or pACT2 with the indicated KAR1 fragments (Gal4-fusions) were co-transformed with pEG202 (no insert) or pEG202 containing SPC72 (lexA-fusions) into strain SGY37. The activity was determined by a plate assay. Blue colony colour indicates interaction. (B) Kar1-1p and Kar1-Δ15p are defective in their interaction with Spc72p. KAR1 (codons 1–408) in pACT2 and SPC72 derivatives in pEG202 were transformed into cells of SGY37. ‘Control’ indicates that the plasmid pEG202 was co-transformed with pACT2–C-SPC72 contains codons 231–622 of SPC72. β-galactosidase activity of lysed cells was measured as described in Materials and methods. Note, it is unclear why the pACT2–C-SPC72 construct gave a stronger signal than pACT2–SPC72. (C) It was established by immunoblotting with anti-HA antibodies that the Gal4–HA–KAR1 (lane 1), Gal4–HA–kar1-Δ15 (lane 2) and Gal4–HA–kar1-1 (lane 3) gene fusions were expressed to similar levels. (D) Binding of recombinant histidine-tagged C-Spc72p to GST–Kar1p, GST–Kar1-Δ15p (lane 3) and GST–Kar1-1p (lane 4) proteins bound to glutathione–Sepharose were incubated with an E.coli extract containing 6His–C-Spc72p (lane 5). Bound proteins were analysed by SDS–PAGE and Coomassie Blue staining, and immunoblotting using anti-C-Spc72p antibodies. 6His–C-Spc72p bound to GST–Kar1p (lane 2) was already detectable in the Coomassie Blue stained gel (asterisk). The molecular masses of the standard proteins are indicated in kDa. (E) SPC72 is a high dosage suppressor of the mating defect of kar1-1 cells. Cells of KAR1 (lanes 1 and 2; ESM643-1/2) and kar1-1 (lanes 3 and 4; ESM614-1/2) carrying either pRS426 (lanes 2 and 4) or SPC72 on the multi-copy vector pRS426 (pRS426–SPC72, lanes 1 and 3) were crossed with wild-type cells (strain JY431). Karyogamy efficiency was determined as described in Materials and methods.

Spc72p-GFP signal (Figure 2A, lower). We established that the karyogamy deficiency and the Spc72p localization defect of kar1-Δ15 cells were complemented by a plasmid carrying KARI and that the SPB in the kar1-Δ15 cell body was labelled when the central plaque component Spc42p-GFP (Bullitt et al., 1997) was used as SPB marker (data not shown). Thus, in mating cells the association of Spc72p with the SPB depends on the N-terminal domain of Kar1p.

It has been shown that deletion of the outer plaque component Cnm67p impairs CM organization by the outer plaque. In these cells all CMs originate from the half bridge (Brachat et al., 1998; illustrated in Figure 8C). Since Spc72p is associated with the outer plaque (Knop and Schiebel, 1998; Wigge et al., 1998) and in a Kar1p-dependent manner with the half bridge (Figure 2A and B), Spc72p should not be associated with the SPB in the kar1-Δ15 Δcnm67 double mutant and these cells should hardly be viable, because Spc72p fails to bind to the SPB and therefore is unable to fulfil its essential function in our strain background (Chen et al., 1998; Knop and Schiebel, 1998). It is important to note that for unknown reasons SPC72 is not essential in some other strains (Souès and Adams, 1998). The viability of cells carrying the kar1-Δ15 Δcnm67 mutations was investigated using a plasmid shuffle approach (Figure 2C). In agreement with a previous report (Vallen et al., 1992), kar1-Δ15 cells grew as wild-type [compare sector 2 (kar1-Δ15) with 1 (wild-type)]. In contrast, Δcnm67 cells (sectors 3 and 4) showed a growth defect (Brachat et al., 1998) which was increased further by the kar1-Δ15 mutation, indicated by the fact that no Δcnm67 kar1-Δ15 colonies were obtained after 3 days.
The molecular role of Kar1p in karyogamy (sectors 5 and 6). However, rare survivors were observed after 6 days which were tested for the localization of Spc72p (Figure 2D). Whereas Spc72p was associated with nearly all SPBs (>97%, n = 120) in wild-type (KAR1 CNM67), kar1-Δ15 (kar1-Δ15 CNM67) and Δcnm67 (KAR1 Δcnm67) cells throughout the cell cycle (Figure 2D, compare the anti-Spc98p signals with the anti-Spc72p signals), it was not detectable at the SPB in kar1-Δ15 Δcnm67 cells. Instead, Spc72p was visualized as a few dots in cells with one DAPI staining region (Figure 2D, upper large budded cell in kar1-Δ15 Δcnm67) or in cells with multiple nuclei (Figure 2D, lower cell in kar1-Δ15 Δcnm67) and these dots did not co-localize with the anti-Spc98p signals, representing the SPBs. The anti-Spc72p dots may represent Spc72p polyomers, which might form due to the self-assembly properties of Spc72p (Souè and Adams, 1998). Cells of kar1-Δ15 Δcnm67 were also found to be packed with up to 15 nuclei (Figure 2D, large budded cell on the bottom left), possibly caused by a severe nuclear migration failure due to the lack of CMs. Taken together, the SPB localization of Spc72p in vegetatively growing cells is dependent on a functional outer

[Diagram A: DAPI, Texas-Red, Spc72p-GFP, Phase]

[Diagram B: KARI, kar1-Δ15, Phase, Spc72p-GFP]

[Diagram C: Circular array with sectors 1 to 6]
plaque and on the N-terminal domain of Kar1p throughout the cell cycle. In contrast, in mating cells the SPB localization of Spc72p is solely dependent on Kar1p.

Our data indicate that Spc72p is associated with the outer plaque and via Kar1p with the half bridge of the SPB (Figure 2D). In kar1-Δ15 cells Spc72p is probably only associated with the outer plaque. Therefore, the disappearance of the Spc72p signal in kar1-Δ15 cells after the addition of pheromone suggests that the amount of Spc72p at the outer plaque of the SPB is decreased drastically in response to mating pheromone (Figure 2A and B). However, it is unclear whether Spc72p at the half bridge is regulated by the pheromone pathway and whether Spc72p fluctuates within the SPB in a cell-cycle-dependent manner during vegetative growth. To investigate this, the fluorescence intensity of 20 SPBs of SPC72-GFP cells was determined. We found that the SPB signal of α-factor arrested wild-type cells (relative fluorescence intensity of 6.7 ± 1.7) was higher compared with cells in G1 (4.7 ± 2.3) or in mitosis (4.7 ± 1.6). This increased Spc72p-GFP signal observed in α-factor arrested cells, in which Spc72p is solely associated with the half bridge via Kar1p (Figure 2A and B), not only indicates that in response to pheromone Spc72p dissociates from the outer plaque but also that more Spc72p binds to the SPB half bridge. In addition, we followed the Spc72p-GFP signal in kar1-Δ15 cells, in which Spc72p is only associated with the outer plaque. In these cells the Spc72p-GFP signal was lower in G1 (1.3 ± 0.5) compared with mitosis (4.0 ± 3.0) and no signal above background was observed.

**Fig. 2.** *In vivo* binding of Spc72p to the SPB requires Kar1p and Cnm67p during vegetative growth, but is only dependent on Kar1p in mating cells. (A) The SPB localization of Spc72p during conjugation is dependent on Kar1p. SPC72-GFP wild-type cells (ESM617) were marked with Concanavalin A conjugated to Texas Red. The washed cells were then incubated for 1–3 h at 30°C with SPC72-GFP KARI (ESM504) or SPC72-GFP kar1-Δ15 (YMK232) cells to obtain intermediates of the karyogamy process. Spc72p-GFP in paraformaldehyde-fixed cells was visualized by fluorescence microscopy. DNA was stained with DAPI. Cells were also inspected by phase-contrast microscopy. Two yeast cells are shown whose cell bodies have fused, however, karyogamy has not occurred. Spc72p-GFP is associated with both SPBs when two wild-type cells were crossed (upper panel). Spc72p-GFP was only associated with the SPB of the wild-type cell (marked with concanavalin Texas Red) when a wild-type cell was incubated with and without α-factor (red arrow). Cells incubated with and without α-factor were mixed in order to compare the Spc72p-GFP signals. Panels 6 and 7 show different planes of the same cells. The white arrows in 2, 4, 6 and 7 point towards Spc72p-GFP signals. Bar in B5: 3.5 μm. (B) Binding of Spc72p to the SPB in mating cells is dependent on the Spc72p-binding domain of Kar1p. SPC72-GFP wild-type (ESM504) and SPC72-GFP kar1-Δ15 (YMK232) cells were incubated with or without α-factor for 2 h at 30°C. Cells were inspected by phase-contrast and fluorescence microscopy (Spc72p-GFP). Panels 1–4 display wild-type cells with (3 and 4, black arrow) or without (1 and 2) α-factor. Cells incubated with and without α-factor were mixed in order to compare the Spc72p-GFP signals. Panels 6 and 7 show different planes of the same cells. The white arrows in 2, 4, 6 and 7 point towards Spc72p-GFP signals. Bar in B5: 3.5 μm. (C) Deletion of Cnm67 and the kar1-Δ15 mutation are synthetically lethal. A strain was constructed which was chromosomally deleted in Cnm67 and which carried Cnm67 on the URA3-based plasmid pRS316 (ESM431). KARI of ESM431 was replaced by kar1-Δ15 (YMK207). Growth of cells of ESM431 with the LEU2-based plasmid pRS315 (KARI Δcnnm67, sectors 3 and 4) or Cnm67 on pRS315 (KARI Cnm67, sector 1) was tested for 3 days at 30°C on plates containing 5-Fluoroorotic acid (5-FOA) which selects against the URA3-based plasmid pRS316-Cnm67. Similarly, cells of YMK207 with pRS315 (kar1-15 Δcnnm67, sectors 5 and 6) or with Cnm67 on pRS315 (kar1-15 Cnm67, sector 2) were grown on 5-FOA. The failure of ESM431 (pRS315) cells to grow on 5-FOA (sectors 5 and 6) indicates synthetic lethality of Δcnnm67 and kar1-Δ15. (D) Localization of Spc72p with the SPB is dependent on the N-terminal domain of Kar1p and on Cnm67p. Wild-type cells (KARI Cnm67, YPH499) and cells of Δcnnm67 (KARI Δcnnm67, ESM424), kar1-Δ15 (kar1-Δ15 Cnm67, YMK179) and Δcnnm67 kar1-Δ15 (ESM658) were investigated by indirect immunofluorescence with anti-Spc98p and anti-Spc72p antibodies. DNA was stained with DAPI. The anti-Spc98p and anti-Spc72p signals were merged. The yellow colour of the merged signals indicates co-localization. Bar, 6.0 μm.
The molecular role of Kar1p in karyogamy

A SPC721–276–KAR1192–433 hybrid functions for SPC72 and KAR1

Our results suggest that the main function of Kar1p in CM organization is to recruit the Tub4p-complex-binding protein Spc72p to the half bridge. Spc72p may then interact with the Tub4p complex which organizes CMs. These functions of Kar1p and Spc72p are also fulfilled by a SPC721–276–Kar1192–433 fusion protein consisting of the Tub4p-complex-binding site of Spc72p (amino acids 1–276) and amino acids 192–433 of Kar1p that target Kar1p to the half bridge (illustrated in Figure 3A). Since the SPC721–276–Kar1192–433 fusion protein contains the domain of Kar1p required for its essential function in SPB duplication (Vallen et al., 1992), this protein may function not only for Spc72p but also for Kar1p.

A plasmid shuffle experiment revealed that the SPC721–276–Kar1192–433 gene fusion expressed from the Kar1p promoter complemented the essential functions of Kar1p (Figure 3B; Δkar1, sector 4), of SPC72 (Δspc72, sector 4) and of SPC72 and Kar1Δkar1 or Δspc72, sector 5). For the further analysis, SPC721–276–KAR1192–433 was integrated into a chromosomal location. The SPC721–276–KAR1192–433 protein was located at the SPB in Δspc72 SPC721–276–KAR1192–433 (Figure 3C, –α-factor) and in Δspc72 Δkar1 SPC721–276–KAR1192–433 cells (Figure 3D, –α-factor) throughout the cell cycle and in cells exposed to pheromone (Figure 3C and D, +α-factor). Quantitation of the SPB signals revealed that the amount of SPC721–276–KAR1192–433 at the SPB was approximately similar to Spc72p (data not shown). Furthermore, the analysis of 15 complete serial sections through SPBs of large budded Δspc72 SPC721–276–KAR1192–433 (Figure 4B and C) or Δkar1 Δspc72 SPC721–276–KAR1192–433 cells (Figure 4E) did not reveal CMs connected with the outer plaque in any of the sections as was the case in all 10 inspected large budded wild-type (Figure 4A) and kar1Δ15 cells (data not shown). Instead, in the mutant cells all CMs originated from the half bridge (Figure 4B, C and E). This analysis shows that CMs are organized differently after α-factor treatment. This result is consistent with the notion that during vegetative growth the amount of Spc72p at the outer plaque decreases in G1 of the cell cycle. However, this decrease is less pronounced compared with the total dissociation of Spc72p from the outer plaque in response to a pheromone signal.

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in large budded Δspc72 SPC721–276–KAR1192–433 cells with or without KARI in comparison with wild-type. In contrast, the organization of CMs was similar in small budded Δspc72 SPC721–276–KAR1192–433 (Figure 4D) and wild-type cells (data not shown; Byers, 1981). In both cell types the CMs started from the bridge (Figure 4D), which connects the two side-by-side SPBs. Taken together, these data suggest that the Spc721–276–Kar1192–433 protein fulfills the functions of Spc72p and of Kar1p at the half bridge and bridge of the SPB.

**CMs at the half bridge can fulfill most of the functions of the CMs of the outer plaque**

Cells of Δspc72 Δkar1 SPC721–276–KAR1192–433 have no CMs at the outer plaque (Figure 4) and therefore lack the major CM array of wild-type cells. Owing to this unique property, we were able to address whether CMs of the half bridge can fulfill the functions of the outer plaque microtubules. Analysis of the microtubules of vegetatively growing cells with SPC721–276–KAR1192–433, but without KARI, SPC72 or KARI SPC72, by indirect immunofluorescence revealed that >93% of these cells had normal microtubule arrays (Figure 5A, Table I). These cells formed a functional mitotic spindle (white arrows) which was aligned along the mother-to-bud axis by the CMs (yellow arrows). The CMs extended from the SPB to the cell cortex and their appearance was as in wild-type cells. In addition, the chromosomes were segregated in cells with a large bud and the nucleus had migrated into the bud. Phenotypes such as two nuclei in the mother cell body or a misaligned mitotic spindle, which are caused by a malfunction of the CMs (Sullivan and Huffaker, 1992; Eschel et al., 1993; Li et al., 1993; Knop and Schiebel, 1998), were only observed in a few cells (Figure 5A, red arrows; Table I). Similarly, when any of these mutant cells were treated with pheromone most CMs appeared as in wild-type cells (Figure 5B; Table II). A broad bundle of nuclear microtubules (white arrow) and a fainter array of CMs (yellow arrow), separated by the SPB in the nuclear envelope, were aligned along the length axis of the cell. However, some pheromone-arrested mutant cells had abnormal CMs (Table II). In these cells, CMs were either not connected with the SPB (Figure 5B, yellow asterisk), a phenotype which was most prominent in Δkar1 SPC721–276–KAR1192–433 cells (Table II), or were not aligned along the length axis of the cell (Figure 5B, white and red asterisks).

Defects in CM organization affect the efficiency of karyogamy during conjugation (Rose and Fink, 1987). However, Δspc72 SPC721–276–KAR1192–433 cells were not karyogamy deficient when crossed to wild-type cells [Figure 5C, bar 1 (wild-type), bar 6 (Δspc72 SPC721–276–KAR1192–433)] and only a very moderate mating defect was observed by crossing both mutant cell types (Figure 5C, bar 3), which was ~1/40 of the defect when kar1 ΔA15 cells were crossed with each other (zygote/diploid ratio of 80). Cells of Δspc72 Δkar1 SPC721–276–KAR1192–433 (Figure 5C, bars 4 and 7) behaved similar to Δspc72 SPC721–276–KAR1192–433 cells (bars 3 and 6). Despite the occurrence of free CMs in some pheromone-treated Δkar1 SPC721–276–KAR1192–433 cells (Table II), these cells had hardly any karyogamy defect either when crossed to wild-type (Figure 5C, bar 5) or to mutant cells (bar 2). In summary, CMs attached to the half bridge via the Spc721–276–Kar1192–433 protein fulfill most of the functions of the outer plaque microtubules during vegetative growth.

**Fig. 4.** The CMs of Δspc72 SPC721–276–KAR1192–433 cells originate at the half bridge of the SPB. (A) SPB of a large budded wild-type cell (YPH499) with CMs attached to the outer plaque. (B) A SPB of a large budded Δspc72 SPC721–276–KAR1192–433 cell (UGY51) is shown with a central plaque and a half bridge and the CMs originate from the half bridge. (C) Two consecutive sections through one SPB of a Δspc72 SPC721–276–KAR1192–433 cell with a large bud are shown. The section on the left-hand side shows the half bridge with CMs. The next section reveals the central and outer plaque of the SPB and the outer plaque is not associated with CMs. Other sections through this SPB did not reveal CMs originating from the outer plaque (data not shown). The asterisk in (C) indicates a nuclear pore. (D) A small budded Δspc72 SPC721–276–KAR1192–433 cell in which the SPB has just duplicated. The two side-by-side SPBs are connected via the bridge. The bridge, but not the outer plaques, is associated with CMs. (E) Two sections through a SPB of a large budded Δkar1 Δspc72 SPC721–276–KAR1192–433 cell (UGY64). The left-hand side shows the central and outer plaque of the SPB and no CMs are associated with the outer plaque. The next section reveals the half bridge which is associated with CMs. Other sections through this SPB did not reveal CMs originating from the outer plaque (data not shown). Abbreviations: B, bridge; CM, cytoplasmic microtubules; CP, central plaque; HB, half bridge; NE, nuclear envelope; NM, nuclear microtubules; OP, outer plaque.
The molecular role of Kar1p in karyogamy

Fig. 5. The CMs of SPC721–276–KAR1192–433 cells that originate from the half bridge are functional. (A, B) Wild-type cells (YPH499) and cells of SPC721–276–KAR1192–433 without SPC72 (UGY51), KAR1 (UGY46) or SPC72 and KAR1 (UGY64) were grown in YPDA medium at 30°C to a density of $5 \times 10^6$ cells/ml. A portion of the cells were used directly for indirect immunofluorescence (A, log cells), whereas the rest was incubated further with α-factor for 2 h at 30°C followed by indirect immunofluorescence with anti-tubulin antibodies (B, α-factor). DNA was stained with DAPI. Cells were inspected by phase-contrast microscopy. (A) The yellow, white and red arrows point towards CMs, the mitotic spindle or cells with a nuclear migration defect, respectively. (B) The nuclear microtubules are marked with a white arrow, whereas the yellow arrows point toward CMs. The yellow, white and red asterisks highlight CMs which are defective. (A) Bar, 5 μm. (B) Bar, 2.5 μm. (C) Cells of Δkar1 Δspc72 SPC721–276–KAR1192–433 show only a weak karyogamy defect. Karyogamy efficiency of wild-type and mutant cells was determined as described in Materials and methods. Shown is the mean value of three experiments. Bar 1, wild-type (ESM670) crossed to wild-type (ESM673); bar 2, both cell types (UGY55 and UGY58) were SPC721–276–KAR1192–433 Δkar1; bar 3, both cell types (UGY56 and UGY59) were SPC721–276–KAR1192–433 Δspc72; bar 4, both cell types (GPY134 and UGY78) were SPC721–276–KAR1192–433 Δkar1 Δspc72; bar 5, wild-type (ESM673) crossed to SPC721–276–KAR1192–433 Δkar1 (UGY55); bar 6, wild-type (ESM673) crossed to SPC721–276–KAR1192–433 Δspc72 (UGY56); bar 7, wild-type (ESM673) crossed to SPC721–276–KAR1192–433 Δkar1 Δspc72 (GPY134).

ative growth. In addition, since the karyogamy defect was small, we conclude that the CMs of SPC721–276–KAR1192–433 cells are also functional during conjugation.

Spc72p, Kar1p and components of the Tub4p complex are required to maintain the CMs at the half bridge

We investigated whether components of the Tub4p complex (Tub4p, Spc98p and Spc97p) and proteins that anchor the Tub4p complex to the half bridge (Kar1p and Spc72p) are required to maintain the CMs after they have been reorganized in response to pheromone. Conditional lethal kar1Δ17, spc72–7, spc97–20, spc98–13 and tub4–1 mutant cells were incubated with α-factor at the permissive temperature (23°C), followed by a short incubation at the restrictive temperature (37°C) in the presence of pheromone.

Wild-type cells exposed to pheromone showed a faint bundle of CMs that was directed from the SPB into the mating projection (Figure 6, yellow arrow), whereas the
Table I. Phenotypes of cells expressing the SPC72\(^{1-276} \cdot \text{KAR1}^{192-433}\) gene fusion

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Cells were grown to early logarithmic phase and prepared for fluorescence microscopy as described in Materials and methods. Approximately 150 cells were counted. The shaded area within the cells indicates the DAPI staining. Values are given in percentages.

Table II. Phenotypes of the pheromone-treated cells expressing the SPC72\(^{1-276} \cdot \text{KAR1}^{192-433}\) fusion gene

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<td>1</td>
<td>1</td>
<td>5</td>
</tr>
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 Cultures at a density of \(5 \times 10^6\) cells/ml were treated with \(\alpha\)-factor for 2.3 h at 30°C. Cells were prepared for indirect immunofluorescence with anti-tubulin antibodies as described in Materials and methods. Approximately 150 cells with a mating projection were counted. Values are given in percentages. The dot indicates the SPB. The bar in the nucleus represents the nuclear microtubules. The thin lines denote the position of the CMs.

broader nuclear microtubules (white arrows) were aligned along the length axis of the cell (black arrow) and overlapped with the DAPI staining region. Frequently, we observed a gap in between the two microtubule bundles (pink arrow), reflecting the localization of the SPB. Both microtubule bundles were present in 97% of the wild-type cells when incubated at 37°C.

We observed that \(>95\%\) of CMs of the pheromone-treated mutant cells appeared normal at the permissive temperature. In contrast, most CMs of \(spc72-7\) (80%), \(spc97-20\) (52%) and \(spc98-13\) (87%) cells were no longer detectable after an incubation at the restrictive temperature, indicating that the encoded proteins have an important role in maintaining the CMs in mating cells (Figure 6).

The effect of the \(tub4-1\) mutation was not as severe, but still 25% of the \(tub4-1\) cells lacked CMs, which is clearly above the wild-type level of 3%. In cells carrying the \(kar1-\Delta 17\) mutation, which causes a temperature-sensitive growth defect (Vallen et al., 1992), detached microtubules were observed in \(~15\%\) of the cells (Figure 6, yellow arrow) and most of the remainder showed misaligned or no (5%) CMs. Furthermore, the nuclear microtubules of cells lacking or having detached CMs were not aligned along the length axis of the cell (Figure 6, black arrow).

![Fig. 6. Kar1p, Spc72p and components of the Tub4p complex are required to maintain the CMs after exposure of cells to pheromone. Cells of wild-type (YPH499), spc72-7 (YMK205), spc97-20 (YM51.20), spc98-13 (GPY13-13A), tub4-1 (ESM218) and kar1-\Delta 17 (MS2083) were grown to mid-logarithmic phase in YPDA medium at 23°C. \(\alpha\)-Factor was added and the cells were incubated for 2.5 h at 23°C until \(>95\%\) of the cells showed a mating projection. Cells were resuspended in 37°C pre-warmed YPDA medium containing \(\alpha\)-factor followed by an incubation for 30 min at 37°C. Cells were analysed by phase-contrast microscopy and indirect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. Bar, 2.5 \(\mu\)m.](image)
In contrast, the CMs of pheromone-treated Δcnm67 cells, which lack an outer plaque, were not affected at 37°C (data not shown; Brachat et al., 1998). In summary, Kar1p, Spc72p and components of the Tub4p complex are involved in the maintenance of the CMs in cells treated with mating pheromone.

The Spc721–276–Kar1192–433 protein is part of a complex containing Kar1p and proteins of the Tub4p complex

To obtain biochemical proof for the involvement of the Tub4p complex in CM organization during mating, we tested whether the Tub4p complex is associated with Spc72p in pheromone-treated cells, as this is the case during vegetative growth (Knop and Schiebel, 1998). Tub4p, Spc97p and Spc72p were co-immunoprecipitated with Spc97p–3HA using anti-HA antibodies (Figure 7A, lane 1). We also noticed that Spc97p and Spc72p were resolved into multiple bands due to the phosphorylation of these proteins (Pereira et al., 1998, G.Pereira, unpublished). This immunoprecipitation was specific, since it was not observed when SPC97 cells were used (Figure 7A, lane 2). Thus, the Tub4p complex is in association with Spc72p in pheromone-exposed cells.

Attempts to co-immunoprecipitate Kar1p together with either Spc72p–3HA or Spc97p–3HA were not successful (data not shown). We reasoned that the Kar1p–Spc72p interaction may be destabilized as soon as cells are homogenized or during immunoprecipitation. If this is the case, we should be able to co-immunoprecipitate the Spc721–276–Kar1192–433 protein together with the Tub4p complex. Indeed, we found that in extracts from vegetatively growing Δkar1 Δspc72 SPC721–276–KAR1192–433–6HA cells, the components of the Tub4p complex (Figure 7B, lane 1) were co-immunoprecipitated with the Spc721–276–Kar1192–433–6HA protein (Figure 7B, lane 7). This precipitation was dependent on the HA-tag on the Spc721–276–Kar1192–433 protein (Figure 7B, lane 7). Thus, the Spc721–276–Kar1192–433 protein is associated with the Tub4p complex.

This result was confirmed using an extract from vegetatively growing KAR1–3HA Δspc72 SPC721–276–KAR1192–433 cells (Figure 7C). The Spc721–276–K Kar1192–433 protein (anti-Spc72b blot), the components of the Tub4p complex and Kar1p–3HA were immunoprecipitated by the anti-HA antibodies (Figure 7C, lane 1) and this was not observed when an extract from KAR1 Δspc72 SPC721–276–KAR1192–433 cells was used (lane 2). Taken together, in KAR1 Δspc72 SPC721–276–KAR1192–433 cells, Kar1p is in a larger complex with the Spc721–276–KAR1192–433 protein and the Tub4p complex. In addition, the co-immunoprecipitation of the Spc721–276–Kar1192–433 protein with the half bridge component Kar1p (Spang et al., 1995) further supports our notion that the Spc721–276–Kar1192–433 protein is associated with the half bridge of the SPB.

Discussion

Spc72p at the half bridge has only a minor role during vegetative growth

The budding yeast SPB organizes two spatially distinct CMs arrays. CMs are attached to the outer plaque of the

Fig. 7. Co-immunoprecipitation of SPC721–276–KAR1192–433 protein and components of the Tub4p complex together with Kar1p-3HA. (A) Co-immunoprecipitation of Spc72p, Tub4p, Spc97p and Spc98p. SPC97 cells (GPY64; lane 2) and cells of SPC97–3HA (GPY65; lane 1) were treated with α-factor for 2.5 h at 30°C until >95% of the cells showed a mating projection. Spc97p–3HA was immunoprecipitated using anti-HA antibodies (12CA5). Spc97p, Spc98p, Tub4p and Spc72p in the immunoprecipitate were determined by immunoblotting using affinity-purified antibodies. (B) Co-immunoprecipitation of the SPC721–276–KAR1192–433–6HA protein with Spc97p, Spc98p and Tub4p. Lysates of vegetatively growing Δkar1 Δspc72 SPC721–276–KAR1192–433–6HA (UGY80; lane 1) or Δkar1 Δspc72 SPC721–276–KAR1192–433 cells (UGY80; lane 2) were incubated with anti-HA antibodies and incubated with protein G beads. The Spc721–276–KAR1192–433–6HA protein (anti-Spc72b blot), Spc97p, Spc98p and Tub4p were detected in the immunoprecipitate using anti-N-Spc72p, anti-Spc97p, anti-Spc98p or anti-Tub4p antibodies. (C) Co-immunoprecipitation of Kar1p-3HA with the Spc721–276–KAR1192–433 protein, Spc97p, Spc98p and Tub4p. Lysates from cells of KAR1–3HA Δspc72 SPC721–276–KAR1192–433 (GPY115; lane 1) or KAR1 Δspc72 SPC721–276–KAR1192–433 (UGY59; lane 2) were incubated with anti-HA antibodies. The indicated proteins were detected in the immunoprecipitate by immunoblotting.
SPB throughout the cell cycle, whereas in G₁ of the cell cycle additional CMs originate from the half bridge of the SPB (Byers and Goetsch, 1975). Recently, we showed that the Tub4p complex is targeted to the outer plaque of the SPB via its binding to Spc72p (Knop and Schiebel, 1998; Figure 8A), providing some molecular understanding for how these microtubules are organized. Here we present evidence that Spc72p is not only associated with the outer plaque, but also interacts with the N-terminal domain of the half bridge component Kar1p (Figures 1 and 2). Therefore, it is possible that during vegetative growth Kar1p targets Spc72p to the half bridge and that Spc72p then recruits the Tub4p complex to this SPB substructure to initiate the formation of microtubules. This model is further supported by the observation that during vegetative growth the Spc72p-ΔΔ–Kar1p192–433 protein and the Tub4p complex were co-immunoprecipitated with the half bridge component Kar1p (Figure 7). In addition, while this manuscript was in preparation, Adams and Kilmartin (1999) have shown by immunoelectron microscopy that Spc72p and Tub4p are associated with the SPB half bridge in G₁ cells.

To understand whether the association of Spc72p with the SPB outer plaque and with the half bridge is cell-cycle regulated, we quantitated the Spc72p-GFP signal of SPBs of wild-type and kar1ΔΔ cells. Cells of Δkar1Δ ΔΔ proved particularly useful in this study, because Spc72p is only associated with the outer plaque but not with the half bridge of the SPB as this is the case in wild-type cells (Figure 2A and B). We found that the Spc72p-GFP signal of SPBs of kar1ΔΔ cells decreased by two-thirds when cells progressed from mitosis into G₁, raising the possibility that the association of Spc72p at the outer plaque is to some extent regulated throughout the cell cycle. In contrast, the Spc72p-GFP SPB signal of wild-type cells was the same in G₁ and in mitosis. We propose that in wild-type cells a decrease of Spc72p in G₁ at the outer plaque is balanced by an increase of Spc72p at the half bridge. Such a cell-cycle-dependent fluctuation of Spc72p and Tub4p within the SPB was also most recently suggested based on immunoelectron microscopy (Adams and Kilmartin, 1999). It is possible that Spc72p shows a similar cell-cycle distribution within the SPB as the CMs (Byers and Goetsch, 1975). How the distribution of Spc72p within the SPB is regulated throughout the cell cycle is unclear, but we noted that Spc72p phosphorylation is decreased in G₁ of the cell cycle (G.Pereira, unpublished).

Using the kar1ΔΔ and Δcnm67 mutations, which affect the CMs of either the half bridge or the outer plaque (Figure 8B and C), respectively, we addressed whether the two CM arrays have specific functions. Cells of kar1ΔΔ lack the Spc72p-binding domain of Kar1p (Figure 1) and therefore fail to organize the CMs of the half bridge, a conclusion which was confirmed by electron microscopy (data not shown). Despite this deficiency, no obvious defect has been observed in kar1ΔΔ cells during vegetative growth (Vallen et al., 1993; Figure 2C and D). However, a role of the CMs of the half bridge became apparent in Δcnm67 cells, whose viability depends on the CMs organized by Kar1p (Figure 2C). Taken together, these results suggest that the CMs of the half bridge have only a minor function during vegetative growth. Importantly, this does not mean that the CMs of the half bridge cannot fulfill the functions of the CMs of the outer plaque. Instead, we could clearly demonstrate that the CMs of Δspc72 SPC721–276–KAR1192–433 or Δkar1 Δspc72 SPC721–276–KAR1192–433 cells, which originate exclusively
from the half bridge or bridge of the SPB (Figure 4B–E), were of normal appearance during vegetative growth (Figure 5A). Consequently, functional deficiencies, such as a nuclear migration defect, were only found in a small percentage of cells (Table I). This result also suggests that the cell-cycle-dependent distribution of the CMs within the SPB is not essential for many CM-directed processes.

The function of Kar1p during conjugation is to target Spc72p to the half bridge of the SPB

Kar1p has been identified as one of the first genes involved in karyogamy (Conde and Fink, 1976). In this study we provide the first molecular understanding of the role of Kar1p during conjugation. We show that Kar1p recruits Spc72p to the half bridge in response to a pheromone signal, and this is the major function of Kar1p during karyogamy. Our conclusion is based on the following findings. First, Spc72p interacts with a region in Kar1p which has been implicated as the domain of Kar1p that functions in karyogamy (Conde and Fink, 1976). Secondly, the interaction of Spc72p with Kar1p is impaired by mutations in Kar1p, such as Kar1-lp (Figure 1B and C), which specifically affect karyogamy. Thirdly, the mating defect of kar1-l cells was in part suppressed by the overexpression of SPC72 (Figure 1E). Finally, a hybrid protein in which the N-terminal of Kar1p has been replaced by the Tub4p-complex-binding domain of Spc72p (Figure 8D; Knop and Schiebel, 1998), and thereby mimics the function of the Kar1p–Spc72p complex, fulfilled the role of Kar1p and Spc72p in mating (Figures 3 and 4; Table II). However, it is important to note that we do not exclude the possibility that Kar1p has additional, probably minor roles during karyogamy. This is suggested by the CM defects which occur in some Δkar1 SPC72-276–KAR192–433 cells when arrested with α-factor (Table II). Kar1p may recruit other proteins to the site of microtubule organization which then stabilize the attachment of the CMs to the half bridge.

Secondly, the interaction of Spc72p with Kar1p is impaired with vegetatively growing G1 cells, suggesting that the association with the SPB is solely dependent on the half bridge component Kar1p (Figure 2A and B). In addition, we observed that the Spc72p-GFP signal at the SPB of α-factor-arrested wild-type cells was higher compared with vegetatively growing G1 cells, suggesting that the amount of Spc72p at the half bridge increases when cells are exposed to mating pheromone. Thus, in response to a pheromone signal more Spc72p binds to the half bridge and significantly less Spc72p is observed at the outer plaque. Importantly, the extent of these changes exceeds the cell-cycle-dependent fluctuations of Spc72p within the SPB. Considering that Spc72p interacts with the Tub4p complex (Knop and Schiebel, 1998) which is involved in microtubule organization (Pereira and Schiebel, 1997), the relocalization of Spc72p within the SPB is possibly the ‘landmark’ event in pheromone-induced reorganization of the CMs.

Other proteins that are directed to the SPB by a mating pheromone signal are the kinesin-related protein Kar3p and its associated protein Cik1p (Meluh and Rose, 1990; Page et al., 1994). Similar to Spc72p, the Kar3p/Cik1p complex changes its localization in response to pheromone. The Kar3p/Cik1p complex is found along the mitotic spindle in vegetatively growing cells (Page et al., 1994; Manning et al., 1999), whereas it is associated with the SPB in a Kar1p-dependent manner and along CMs in pheromone-treated cells (Meluh and Rose, 1990; Page and Snyder, 1992). It is known that transcription of KAR3 and CIK1 is increased in mating cells (Meluh and Rose, 1990; Page and Snyder, 1992; Kurihara et al., 1996), a mode of regulation that has been excluded for SPC72 (G.Pereira, unpublished results). Whether this increased expression of KAR3 and CIK1 is sufficient to target the Kar3p/Cik1p complex to the SPB in a Kar1p-dependent manner is unclear. It is possible that for the Kar3p/Cik1p complex, and also for Spc72p, other regulatory mechanisms play a role. Spc72p, the outer-plaque-binding protein of Spc72p or Kar1p may be regulated by phosphorylation in response to mating pheromone as this is the case for Far1p and the transcription factor Ste12p (Chang and Herskowitz, 1992; Elion et al., 1993).

Spc72p was found to be associated with the Tub4p complex in mating cells (Figure 7A). In addition, using temperature-sensitive mutants, we obtained evidence that Kar1p, Spc72p and the components of the Tub4p complex are still required to maintain the half bridge microtubules after they have been formed in response to a mating pheromone signal (Figure 6). These observations raise the possibilities that either the CMs are stable after their formation and remain attached to the half bridge via the Kar1p, Spc72p and the Tub4p complex or that the CMs are unstable and constantly reformed at the half bridge by a de novo nucleation process. In the first case, the CMs detach from the SPB half bridge in temperature-sensitive spc72, spc97, spc98 and tub4 mutants and the released CMs depolymerize. In the second case, the continuous reformation of the CMs is impaired in the temperature-sensitive mutants. We consider the second possibility less likely, since CMs grow and shorten primarily at the shmoo tip in mating cells, and not at the SPB (Maddox et al., 1999). In addition, the free CMs observed in kar1-Δ17 cells (Figure 6) are most easily explained by the first model, assuming that in kar1-Δ17 cells the CMs detach together with the Tub4p/Spc72p complex, which then stabilizes these microtubules. Thus, our data support a model in which in response to a pheromone signal Spc72p is released from the outer plaque and more Spc72p is targeted to the half bridge via Kar1p. Spc72p then recruits the Tub4p complex to this SPB substructure to induce the formation of CMs. After their formation, these CMs remain attached to the half bridge via the binding of the Tub4p complex to Spc72p and Spc72p to Kar1p (Figure 8A).
**Microtubule reorganization processes**

We have shown that budding yeast has at least two so-called γ-tubulin complex-binding proteins (GTBPs), Spc72p and Spc110p, which target the cytoplasmically assembled Tub4p complex (Pereira et al., 1998) to the sites of microtubule organization within the SPB. Spc110p is the GTBP of the inner plaque, whereas Spc72p has been identified as the GTBP of the outer plaque (Knop and Schiebel, 1997, 1998). The results described in this paper extend the model of how GTBPs work. The same GTBP may be associated with multiple centrosomal substructures or with different microtubule-organizing centres (MTOCs) within one cell. GTBPs may change their distribution within the centrosome in response to external and internal signals by binding to different GTBP-binding proteins such as Kar1p. By this means the microtubules may gain specific properties which enable them to fulfill specialized functions. Our data further suggest that γ-tubulin complexes, GTBPs and GTBP-binding proteins not only have a function in microtubule formation, but also have additional roles in the attachment of microtubules to MTOCs.

**Materials and methods**

**General yeast methods**

Standard protocols were used for growth and transformation of yeast cells. Yeast strains were grown in yeast extract, peptone, dextrose (YPD) medium containing 100 mg/ml adenine (YPDA) or in synthetic complete (SC) medium (Sherman, 1991). Yeast strains used in this study were...
derivatives of $288$ except for strains $E Y 93$, JY425 and JY431 used in the kari1 suppression experiment. Strains and plasmids are listed in Table III. The kari1-A15 mutation was introduced by 'pop-in pop-out' of the integration vector pMK21. The kari1-1 allele was cloned by gap repair using strain EY93. All strains constructed during the course of this study were tested for the presence or absence of Cnm67p, Spc72p, Kar1p and the Spc72-1–276–Kar1192–433 protein by immunoblotting with affinity-purified antibodies and by colony PCR with specific primers. MATa yeast cells were synchronized with $1 \mu g/ml$ (SST7 cells) or $10 \mu g/ml$ (SST1 cells) of synthetic r-factor until $>95\%$ of the cells showed a mating projection.

Two-hybrid interactions

Two-hybrid interactions were determined with derivatives of pACT2 (Gyuris et al., 1993) and pEG202 (Durfee et al., 1993) which were transformed into the reporter strain SGY37 (Geissler et al., 1998). $\beta$-galactosidase activity was determined either by a plate assay with 5-bromo-4-chloro-3-indol-$\beta$-D-galactoside (X-Gal) as a substrate (Geisler et al., 1998) or in Z-buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM $\beta$-mercaptoethanol, pH 7.0) with o-nitrophenyl-$\beta$-D-galacto-pyranoside (ONPG). In the latter case, the activity was normalized to the optical density of the culture (Kaiser et al., 1994).

Immunological techniques, fluorescence and electron microscopy

The relative fluorescence intensity raised by Spc72p-GFP at the SPB was measured at the immediate proximity of the SPB. These measurements were taken in the linear range of our detection system. Immunofluorescence of yeast cells was performed using indirect immunofluorescence with a pool of mouse anti-90 kDa mAb (Rout and Kilmartin, 1990). Secondary antibodies were from Jackson Laboratories. Anti-Kar1p, anti-N-Spc72p, anti-Spc97p, anti-Spc98p and anti-Tub1p antibodies were used to test the immunoprecipitates (Spang et al., 1995, 1996; Geisler et al., 1996; Knop et al., 1997; Knop and Schiebel, 1998). The anti-N-Spc72p antibodies also recognized the Spc72-1–276–Kar1192–433 protein. Antibodies directed against the C-terminal domain of Spc72p were affinity-purified using a histidine-tagged Spc72p-GR2-622.

In vitro binding of C-Spc72p to GST-Kar1p

Spc72-1–276–Kar1192–433 was cloned into the E.coli expression vector pET28c (pMK297). KARI, kari-1 and kari-1A15 were ligated into pGEX-5X-1. pET28c and pGEX-5X-1 derivatives were transformed into E.coli BL21 DE3 (Studier and Moffat, 1986). Recombinant proteins were expressed upon the addition of 0.5 mM isopropyl-$\beta$-D-thiogalacto-pyranoside (IPTG) for 4 h at 30°C. GST fusion proteins were bound to glutathione–Sepharose (Pharmacia) as recommended by the manufacturer. An E.coli extract containing 6His-Spc72-1–276–Kar1192–433 was incubated with the glutathione beads for 1 h at 4°C in phosphate-buffered saline (PBS) containing 0.1% Triton-X100. The resin was washed once with PBS, 0.1% Triton-X100, followed by two washes with PBS. Bound proteins were eluted with SDS-sample buffer and analysed by Coomassie Blue staining and immunoblotting with anti-C-Spc72p antibodies.

Table III. Continued

<table>
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<tr>
<th>Name</th>
<th>Genotype</th>
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*SPC72-1–622 indicates that codons 1–622 of SPC72 have been cloned into plasmid pEG202.*
Quantitative mating

Yeast strains with the genotypes MATa Ura+ trp1 (EM670, UGY55, UGY56, GPY134) or MATα Ura3-52 Trp+ (EM673, UGY58, UGY59, UGY70) were grown at 30°C in SC medium lacking uracil or tryptophan, respectively, to a density of 10^7 cells/ml. Cells (3 × 10^6 cells) of both mating types were mixed and filtered onto a nitrocellulose membrane. The membrane was placed onto a YPD plate which was incubated for 4 h at 30°C. Cells were washed with PBS and dilutions were plated onto YPD plates (number of viable cells) and SC plates lacking tryptophan or uracil (number of diploid cells). Both plates were incubated for 3 days at 30°C. The percentage of diploid cells was determined. The cells in PBS were also inspected using light microscopy to determine the percentage of zygotes in the suspension. In the case of the kar1-1 suppression analysis by SC72, MATA Ura2-11S Trp+ cells (EM663-1/2; EM664-1/2) with plasmids pRS426 or pRS426-SC72 were grown and stored in SC medium lacking uracil to a density of 10^7 cells/ml. JY431 (MATa trplA1 Leu⁵) cells were grown in YPD medium at 30°C. After mating, cells were plated onto YPD plates and SC plates without leucin and tryptophan to determine the percentage of diploid cells.

Acknowledgements

This work was supported by a project grant (Ref. SP 2496/0101) from the Cancer Research Campaign. We thank Dr F.Barr for comments on the manuscript. Dr J.Kilmartin is acknowledged for the 90-kDa antibodies and M.O’Prey for the help with the electron microscopy.

References


