Components of the Yeast Spindle and Spindle Pole Body

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Abstract. Yeast spindle pole bodies (SPBs) with attached nuclear microtubules were enriched ~600-fold from yeast cell extracts. 14 mAbs prepared against this enriched SPB fraction define at least three components of the SPB and spindle. Immunofluorescent staining of yeast cells showed that throughout the cell cycle two of the components (110 and 90 kD) were localized exclusively to the SPB region, and the other (80 kD) was localized both to the SPB region and to particulate dots in short spindles. Immunoelectron microscopy confirmed and extended most of these findings. Thus the 110-kD component was localized to a layer in the SPB just to the nuclear side of the plane of the inner nuclear membrane. The 90-kD component was localized in a layer across the cytoplasmic face of intact SPBs, and, in SPBs where nuclear microtubules were removed by extraction with DEAE-dextran, the 90-kD component was also found in an inner nuclear layer close to where spindle microtubules emerge. In intact SPBs with attached nuclear microtubules the anit-80-kD mAb labels microtubules, particularly those close to the SPB. These results begin to provide a preliminary molecular map of the SPB and should also enable the corresponding genes to be isolated.

SPINDLE pole bodies (SPBs)[1] are cylindrical multilaminate organelles embedded in the nuclear envelope that are responsible for the organization of cytoplasmic, mitotic, and meiotic microtubules in the yeast *Saccharomyces* (Robinow and Marak, 1966; Moens and Rapport, 1971; Byers and Goetsch, 1974). They are the functional analogues of the centrosomes of higher eukaryotes. In thin sections of whole yeast cells, SPBs have two clear structurally distinct plaques or layers (Moens and Rapport, 1971; Byers and Goetsch, 1975): a lightly staining outer cytoplasmic plaque above the nuclear membrane where cytoplasmic microtubules are attached, and a densely staining central plaque in the plane of the nuclear membrane. In addition, negatively stained images of isolated yeast mitotic spindles (King et al., 1982) and thin sections of meiotic cells (Moens and Rapport, 1971; Peterson et al., 1972) show the presence within the nucleus of a third, inner layer or plaque, from which the spindle microtubules appear to originate. During mitosis SPBs initiate both continuous pole to pole microtubules and discontinuous chromosomal microtubules from sealed ends on their nuclear face (Byers et al., 1978), there being one microtubule per chromosome (Peterson and Ris, 1976; King et al., 1982) as in most fungi (Heath, 1978). During mating, the two haploid SPBs fuse (Byers and Goetsch, 1975) to produce the larger diploid SPB, which is then stably replicated at the larger size in diploid cells. This larger size allows the diploid SPB to accommodate the extra chromosomal microtubules needed (Peterson and Ris, 1976), whereas there is only a slight increase in the number of pole-to-pole microtubules (Peterson and Ris, 1976).

1. *Abbreviations used in this paper*: PVP, polyvinylpyrrolidone; SPB, spindle pole body.

At present little is known in molecular terms about how SPBs initiate and control the number of microtubules, or how they replicate themselves to produce a single exact copy before mitosis. Such a molecular explanation of SPB function could start with the identification of SPB components, whose function may then be rapidly established using the powerful new methods developed in yeast genetics (Botstein and Fink, 1988). It seems likely that some SPB components will have homologues in higher eukaryotes since the protein they organize into microtubules, tubulin, is structurally (Neff et al., 1983; Schatz et al., 1986) and functionally (Kilmartin, 1981) similar in both yeast and mammals. In addition, protein kinases that control the start of mitosis are very similar in yeast and vertebrates (Dunphy et al., 1988; Gautier et al., 1988), suggesting the pathways in the formation of the mitotic spindle may also be conserved among eukaryotes.

There are a number of different approaches towards identification of SPB components. The genetic approach has involved the isolation of cell cycle mutants (Hartwell et al., 1973; Morris, 1976; Nurse, 1985; Yanagida et al., 1985), particularly those that may specifically block the formation of the mitotic spindle. One such mutant *cdc 31*, causes a defect in SPB replication resulting in the formation of a single abnormally large SPB (Byers, 1981*a*). The gene codes for a calmodulin-like protein (Baum et al., 1986) and although the immunocytochemistry of this protein has not yet been reported in yeast, homologues are associated with the *Chlamydomonas* basal body (Huang et al., 1988*a*,*b*), suggesting that the gene product of *CDC31* may be associated with the functionally analogous organelle in yeast, the SPB. A different genetic approach is to isolated mutants defective in nuclear fusion during mating (Conde and Fink, 1976; Polania

and Conde, 1982) since this is mediated by SPB fusion (Byers and Goetsch, 1975). One such mutant, *kar1*, is reported to cause abnormalities in SPB structure (Rose and Fink, 1987) and is clearly a candidate for an SPB component. Another gene, *SPA1* (Snyder and Davis, 1988), has been isolated by a different approach, namely screening yeast genomic DNA expression banks with human autoimmune sera containing anti-centrosomal antibodies. Disruptions in *SPA1* cause defects in chromosome segregation, suggesting that it could code for an SPB component. However, immunocytochemical evidence for a direct association of the gene products of either *KAR1* or *SPA1* with the SPB has not yet been published. Immunocytochemistry using antibodies prepared against a fusion protein encoded by another candidate gene, *NSP1*, has been published (Hurt, 1988). The antibodies stain the centrosome in mammalian cells but in yeast stain the nuclear envelope as large patches. These patches overlap the position of the SPB in some but not all cells, suggesting a possible association (Hurt, 1988). However recent immunoelectron microscopy has shown that NSP1 is predominantly associated with the nuclear pore complex in yeast (Nehrbass et al., 1990). In other fungi, an mAb (MPM-2) specific for mitotic phosphoproteins (Davis et al., 1983) stains the SPB region in mitotic cells of both *Aspergillus nidulans* (Engle et al., 1988) and *Schizosaccharomyces pombe* (Masuda et al., 1990). The MPM-2-reactive antigens have not yet been identified. Recently γ-tubulin has been localized to the SPB region in *Aspergillus nidulans* by immunofluorescence, and may form part of the attachment site of microtubules to the SPB (Oakley et al., 1990).

This paper describes a different approach towards identification of SPB components. We developed a specific and fast assay for SPBs based on their ability to assemble brain microtubules (Byers et al., 1978; Hyams and Borisy, 1978), and using this assay devised methods to enrich SPBs ~600-fold. We used this enriched SPB fraction to prepare mAbs and identified three SPB components, showing their close association with the SPB by immunofluorescence microscopy and their localization to discrete regions of the SPB by immunoelectron microscopy.

Materials and Methods

SPB Assay

SPBs were assayed by their specific assembly of brain microtubules (Byers et al., 1978; Hyams and Borisy, 1978), to produce small asters that could be counted by dark-field light microscopy (Kilmartin, 1981). Bovine brain 6S tubulin (Sloboda et al., 1976) was prepared using a Bio-Rad Laboratories (Watford, England) Bio-Gel A5m column. SPBs (0.1 μl, measured by external calibration of 1-5-μl glass micropipettes) were added to 5 μl of ~6 mg/ml 6S tubulin in 0.1 M Na-Pipes pH 6.9, 0.1 mM $MgCl_2$, 1 mM GTP, and the mixture placed on a slide and incubated at 37°C. Brain microtubules grew rapidly from the yeast SPBs and were visible (lengths >10 μm) within 1-3 min. The asters visible within a set field on different parts of the slide were counted and the SPB concentration calculated. The specific activity was the total number of SPBs divided by the total amount of protein as measured by the Lowry assay (Schacterle and Pollack, 1973).

Preparation of Yeast Nuclei

Nuclei were prepared by the method of Rozijn and Tonino (1964) with minor modifications using either *S. uvarum* (NCYC 74, a diploid of unknown genotype) or a protease-deficient strain of *S. cerevisiae* BJ2168, (MATa *leu2 trp1 ura3-52 prb1-1122 pep4-5 prc1-407 gal2*) constructed by E. Jones (Carnegie Mellon University, Pittsburgh, PA). Log phase cells (36 liters) were harvested (at 1×10^7/ml for NCYC 74, and 2×10^7/ml for BJ2168), and the cell walls digested in 200 ml 1.1 M sorbitol with 10 ml glusulase (DuPont Corp., Boston, MA), 40 mg zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan) and 30 mg mutanase (Novo Industri, Copenhagen, Denmark) at 30°C for 2-3 h. Spheroplasts (<0.05% intact cells on 20-fold dilution into water) were washed once with 1.1 M sorbitol and centrifuged (2,000 *g*, 15 min, 4°C) through cushions of 7.5% Ficoll 400 (Pharmacia, Uppsala, Sweden) in 1.1 M sorbitol. Spheroplasts were then lysed with a Polytron (Kinematica, Luzern, Switzerland) in 300 ml polyvinylpyrrolidone (PVP) medium (Rozijn and Tonino, 1964) containing a 1:200 dilution of solution P (90 mg PMSF, 1 mg pepstatin A in 5 ml absolute ethanol). All subsequent solutions contained a 1:1,000 dilution of solution P. The crude nuclei pellet was resuspended and adjusted to 1.7 M sucrose-PVP, then overlayed onto three 8-ml layers of 2.0, 2.1, and 2.3 M sucrose-PVP (apparent refractive indices 1.437, 1.442, and 1.454, respectively) in 12 Beckman Instruments (High Wycombe, England) SW28 tubes. The tubes were spun at 28,000 rpm for 4 h at 4°C, nuclei were removed from the 2.1/2.3-M sucrose interface and stored at −70°C.

Preparation of Enriched SPBs

Nuclei from 36 liters of cells were adjusted to 1.9 M sucrose-PVP (apparent refractive index 1.434), divided into four aliquots and pelleted at 120,000 *g* for 1 h in a 70Ti rotor (Beckman Instruments). Nuclear pellets in each tube were lysed by vortexing with 1 ml/1OD 260 nm of 0.1 mM $MgCl_2$, 1.5% Triton X-100, 2.0% digitonin (water soluble, D1407; Sigma Chemical Co., Poole, England), 20% (vol/vol) DMSO, 1:100 solution P, and 20 μg/ml DNase I-EP (Sigma Chemical Co.). After resuspension the frothy solution was allowed to stand for 5 min at room temperature to digest DNA, then an equal volume of 2.5 M sucrose, 10 mM bis-Tris/Cl pH 6.5, 0.1 mM $MgCl_2$ (sucrose-bt) was added and thoroughly mixed. The froth and large particulate material were removed by a brief low-speed spin (3,600 *g*, 6 min) and the supernatant loaded onto three or four pre-cooled Beckman SW28 tubes containing 2.5 ml 2.50 M, 7.5 ml 2.25 M, 5.0 ml 2.00 M, and 5.0 ml 1.75 M sucrose-bt. The tubes were spun at 28,000 rpm, 4°C, 6 h. The gradient layers were unloaded from the top and assayed for SPBs. That part of the gradient spanning the 2.00/2.25 M interface (~7.5 ml/tube, fraction 4, Fig. 2 *A*) was found to contain ~70% of the SPBs applied. These could be stored at −20°C or further enriched by centrifugation in a special gradient of Percoll (Pharmacia). SPBs (2 ml) were first digested with RNase A (20 μg/ml) for 15 min at room temperature, then a cold mixture of 7 ml 2.50 M sucrose-bt, 1 ml Percoll, and 4 ml 10 mM bis-Tris/Cl pH 6.5, 0.1 mM $MgCl_2$, 0.1 mM GTP, 0.1 mM EGTA, 0.1 mM DTT, 20% (vol/vol) DMSO (DMSO buffer) was added. The mixture was placed in a SW28 centrifuge tube (Beckman Instruments) and the remaining two-thirds of the tube carefully topped up with DMSO buffer. The tubes were spun at 28,000 rpm, 6 h, 4°C. After centrifugation a faint white band was visible ~1 cm into the gradient; its position was marked and the gradient unloaded to within 2-3 mm of the band. The next layer, ~4.5 ml (fraction 6, Fig. 2 *A*) was collected right through the band. Fraction 7 was the next 3.5 ml collected, and fraction 8 was the final 2 ml after vortexing to resuspend any pellet. When SPBs were assayed, fraction 6 was found to contain 60-70% of the SPBs originally loaded onto the gradient. Alternatively the fractions could be assayed by SDS gel electrophoresis because the 110-kD and tubulin bands were clearly visible at this stage on SDS gels. The SPBs were concentrated by addition of 3.5 vol of cold 10 mM bis-Tris/Cl pH 6.5, 0.1 mM $MgCl_2$, 20% (vol/vol) DMSO (bt-DMSO) and centrifuged at 120,000 *g*, 1 h, 4°C. The SPBs pelleted as a faint translucent layer on top of a transparent Percoll pellet, and after 5 min on ice, this layer slid off the Percoll pellet to the bottom of the tube. The supernatant was carefully aspirated down to the delicate SPB layer, which was then removed and respun for 410,000 *g*, 1 h, 4°C. The SPBs again pelleted on top of a relatively large Percoll pellet. The SPB layer could be resuspended in a minimal volume of bt-DMSO (or PBS in those fractions used for monoclonal antibody production) either by allowing it to slide off the Percoll pellet into the resuspension buffer or by brief vortexing. The clumps of SPBs were homogenized by gentle sonication in a sonicator bath, and then stored at −20°C.

Preparation of Yeast Tubulin

Yeast tubulin was prepared by a modification of the previous method (Kilmartin, 1981). *S. uvarum* NCYC74 cells (36 liters) were harvested at 2.5×10^7 cells/ml, broken with glass beads and centrifuged (150,000 *g*, 1 h, 4°C) and the supernatant was chromatographed on an 8.6 cm diameter × 3.5 cm long column of DEAE-Sephadex A-50 (Pharmacia). The proteins eluting between 0.2 and 0.5 M KCl were directly precipitated with ammo-

nium sulfate (40.4 g/100 ml), and gel filtered against Pipes assembly buffer (Kilmartin, 1981) to remove KCl and ammonium sulfate. After concentration by ultrafiltration (Amicon Corp., Danvers, MA) to <1 ml, the tubulin was made 5 mM in both DTT and GTP and assembled at 25°C as monitored by dark-field light microscopy. After centrifugation a large translucent pellet was obtained. Microtubules were only a minor part of the pellet, the rest was other polymers which also underwent temperature-dependent assembly-disassembly. However, these contaminating polymers did not appear to require GTP to remain assembled. Thus, tubulin could be separated from them on gradually removing the GTP by washing the pellet with Pipes buffer without GTP and incubating it in this for 5 min at 25°C. When the GTP concentration was sufficiently low, usually in the second or third wash, the microtubules disassembled and the soluble tubulin so released was separated by centrifugation from the polymers, which remained assembled. After addition of 1 mM GTP to each supernatant the microtubules were reassembled (25°C, 1 h) and assayed by dark-field microscopy to discover which tubes contained microtubules. The purified yeast microtubules were pelleted and stored at −70°C.

mAbs

SPBs were resuspended in PBS, emulsified with Freund's complete adjuvant and 5×10^{10} SPBs were injected subcutaneously into each BALB/c mouse. 6 wk later each mouse was boosted with the same dose of SPBs in Freund's incomplete adjuvant. 4 d before the fusion the mouse was injected intravenously with $\sim 5 \times 10^{10}$ SPBs in PBS. The spleen was dissociated by grinding between two sterile ground glass slides then fused to NSO/1 cells and the resultant hybridomas (Galfrè and Milstein, 1981; Goding, 1983) plated into 1000 wells with mouse peritoneal macrophage feeder cells. Supernatants were screened by immunofluorescence of a mixture of SPBs and yeast cells. About 2 μl of SPBs from the 2.00/2.25 M sucrose gradient interface were applied to the wells of a 15-well slide (Flow Laboratories, Irvine, Scotland), aspirated and fixed for 2–3 min with 3.7% (vol/vol) formaldehyde first in 1.5 M sucrose-bt, then in 0.1 M potassium phosphate pH 6.5. Fixed *S. uvarum* cells (Kilmartin and Adams, 1984) were added, followed by fixation in methanol for 5 min at −20°C and acetone for 30 s at −20°C. The cells were included as a focal plane marker and to pick up any other interesting staining patterns. Positive supernatants were also assayed by comparison of nitrocellulose blots of equal protein loadings of 2.00/2.25 M sucrose SPBs and Percoll SPBs separated on SDS gradient polyacrylamide gels. Dried skimmed milk was used as a blocking agent. Antibodies were detected by incubation of the blots with affinity-purified rabbit anti-mouse IgG followed by ^{125}I-labeled protein A. Cells in positive wells were cloned twice by limiting dilution. The Ig subclass of the mAbs was established by staining dot blots of supernatants with anti-mouse IgG or anti-mouse IgM antibodies. All the experiments in this paper were carried out with cell culture supernatants.

Immunofluorescence

Spheroplasts were prepared from log phase (1×10^7 cells/ml) *S. uvarum* cells by digestion with 10% (vol/vol) glusulase and zymolyase 20T (100 μg/ml) in 1.1 M sorbitol for 90 min at 30°C. Spheroplasts were allowed to recover for 30–60 min at 25°C by culture in 1.1 M Sorbitol/Wickermans (2% glucose, 0.5% yeast extract, 0.5% liquid malt extract, and 1% bactopeptone). After washing in 1.1 M sorbitol, spheroplasts were applied to polylysine-coated 15-well slides and fixed in methanol for 5 min at 0 or −20°C, followed by acetone for 30 s at room temperature. Each well was treated for 1 min with fresh 20% (wt/vol) dried skimmed milk followed by the antibody supernatant for 1 h at 30°C or overnight at 4°C. After washing in 2% milk, 0.1% Tween 20 in PBS, affinity purified rabbit antiyeast tubulin was applied for 1 h at 30°C followed by a mixture of affinity-purified goat FITC anti-mouse IgG preabsorbed with rabbit IgG and affinity-purified goat rhodamine anti-rabbit IgG preabsorbed with mouse IgG. After the final wash, milk was removed by washing in 1% BSA-PBS, and the slides were mounted as described by Kilmartin and Adams (1984).

Immunoelectron Microscopy

SPBs from the 2.00/2.25 M sucrose gradient fraction were diluted with 1.5 vol of bt-DMSO and centrifuged (23,000 *g*, 30 min, 4°C) in wells cut from a 96-well microtiter plate as described by Wray and Sealock (1984). The wells were washed with 5% dried skimmed milk, 0.1% Tween-20, 1:1,000 dilution of solution P in bt-DMSO and a 1:5 dilution of antibody supernatant in this milk buffer added for 1 h at 25°C. After washing as above, a 1:20 dilution of 10 nm gold-labeled affinity purified goat anti-mouse IgG (Sigma Chemical Co.) was added in the same buffer for 12 h at 25°C. The wells were washed with milk buffer, then with bt-DMSO to remove milk, and were fixed with 1% (vol/vol) glutaraldehyde for 2 h first in bt-DMSO then in 50 mM potassium phosphate, pH 6.9. Wells were then postfixed with osmium tetroxide, dehydrated with graded ethanol and embedded in Epon.

All gold particle counts and measurements of average positions of gold particles staining the SPB were carried out on SPBs that appeared perpendicular to the direction of sectioning. Under these conditions, the various layers, particularly the intermediate line, were clearly defined.

Chemical Extraction of the SPBs

Heparin extracted SPBs (heparin-SPBs) and DEAE-dextran extracted SPBs (DEAE-SPBs) were prepared by dilution of 1 vol of crude SPBs from the 2.00/2.25 M sucrose gradient fraction into 4 volumes of either bt-DMSO or the same buffer without DMSO, 5 mM mercaptoethanol, 1:500 solution P containing either 1.0 mg/ml heparin or DEAE-Dextran (Pharmacia) respectively. The mixtures were left for 12 h at 0°C then loaded into centrifuge tubes (SW55.1; Beckman Instruments) (2.5 ml per tube) containing 1.0 ml 2.50 M and 1.0 ml 2.00 M sucrose-bt and centrifuged at 50,000 rpm (350,000 *g*) for 3 h at 4°C. The extracted SPBs were recovered from the 2.00/2.50 M sucrose interface, using immunofluorescence microscopy with anti-SPB mAbs as an assay.

Results

The SPB Enrichment Procedure

In developing an enrichment procedure for SPBs, we aimed to fulfill a number of criteria. First, the procedure had to be large scale and of high yield, producing quantities of SPB proteins sufficient to raise antisera in mice for the production of mAbs. Second, it was important that the final fraction should be highly enriched for SPBs to increase the specificity of the immune response from the recipient animals. Third, the morphology of the SPBs as seen by EM (Moens and Rapport, 1971; Byers, 1981b; King et al., 1982) had to be retained as much as possible, while at the same time removing contaminating material such as chromatin and nuclear envelope, to make ultrastructural analysis and immunolocalization of SPB components simpler. Fourth, it was desirable that the endogenous nuclear microtubules attached to the SPBs should be preserved so that tubulin, a minor protein in yeast (Kilmartin, 1981), could act as a marker during enrichment and potential microtubule-associated proteins could also be isolated (Pillus and Solomon, 1986). Furthermore, these endogenous microtubules were needed as the basis for a quick and quantitative biological assay for SPBs, involving the specific polymerization of exogenous brain tubulin off the distal ends of the microtubules. Removal of the endogenous yeast microtubules was always associated with the loss of the ability of SPBs to polymerize brain microtubules, constraining the range of conditions that could be used during the enrichment procedure (Kilmartin and Fogg, 1982). The conditions which best preserved the nucleation capacity of the SPBs as measured by our dark field assay were a salt concentration <50 mM, a pH near 6.5 and the presence of >1.5 M sucrose or 10% DMSO. Except for the requirement for low pH, these conditions are similar to those required for the preservation of centrosome nucleation capacity (Mitchison and Kirschner, 1984; Kuriyama, 1984).

The enrichment procedure as finally developed involved the isolation of nuclei from 36 liters of yeast cell culture by modifications of an established method (Rozijn and Tonino, 1964). SPBs were then released from the nuclei with a mixture of Triton X-100 and digitonin, containing DNase I and

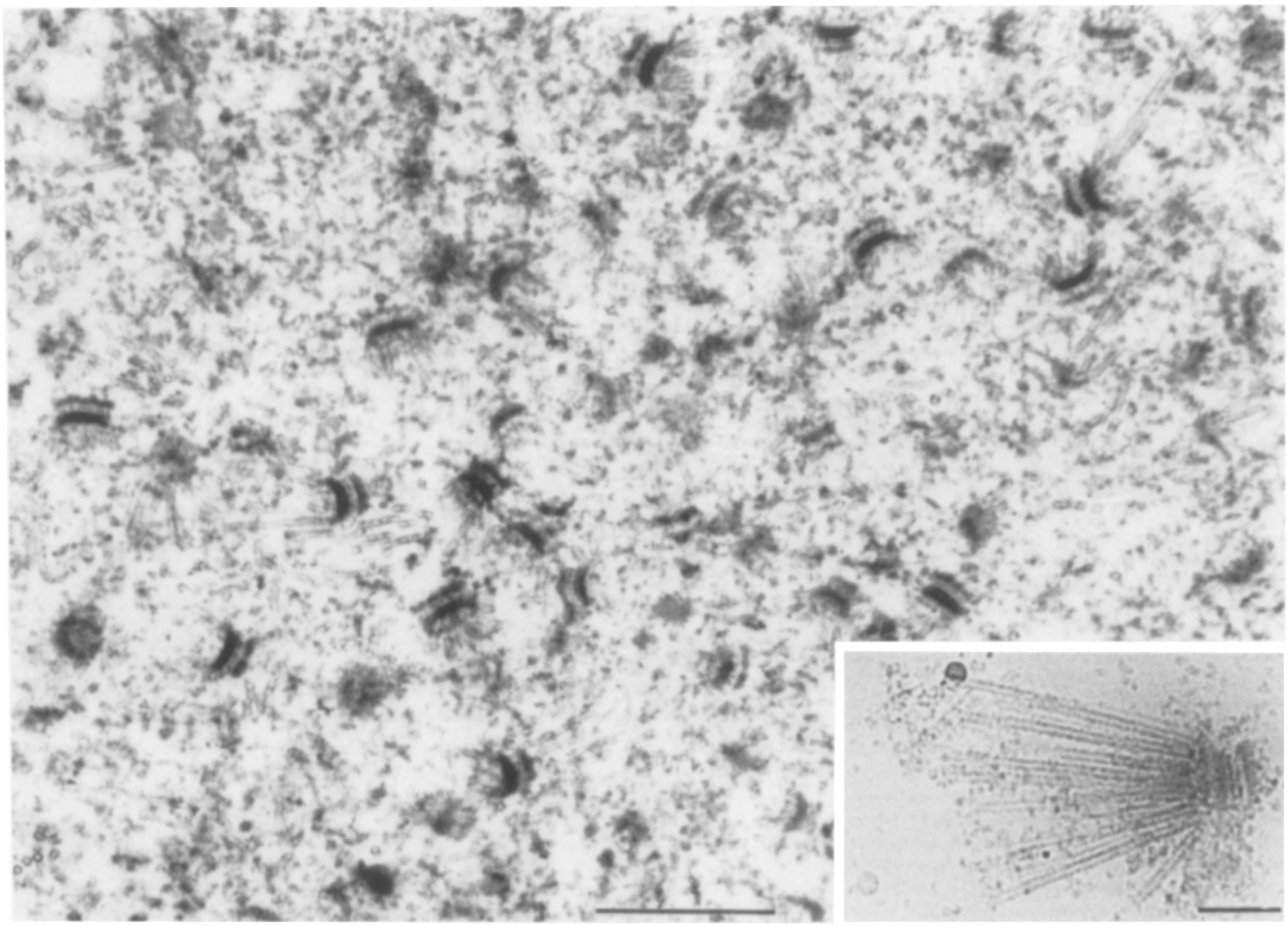

Figure 1. Thin section of a pellet of Percoll gradient-enriched SPBs. Bar, 0.5 μm. (*Inset*) Quick-frozen, unstained view of an SPB by cryo-electron microscopy (experiment performed by C. Akey and N. Unwin). Bar, 0.2 μm.

DMSO under low salt conditions. The rationale for this combination of conditions was that in previous studies on rat liver nuclei, a combination of low salt and DNase I was shown to disperse the internal contents of the nuclei, while retaining the nuclear envelope (Dwyer and Blobel, 1976). We assumed that yeast nuclei would be extracted similarly, and indeed both dark-field light microscopy and EM of negatively stained specimens of yeast nuclei treated with DNase I under low salt conditions in the absence of detergents showed a patch that appeared to be physically associated with the SPB. We cannot yet be sure that this patch was the yeast nuclear envelope because suitable markers for it are not yet available. The patch could be solubilized by a mixture of Triton X-100 and the nonionic steroid detergent digitonin. Ionic cholate detergents were equally effective at solubilization but reduced the biological activity of the SPBs. DMSO was added as a microtubule-stabilizing agent (Himes et al., 1977) since unlike other microtubule-stabilizing agents tested, it gave efficient preservation of yeast microtubules without altering the low viscosity of the lysis solution needed for this procedure.

The SPBs and attached microtubules were then separated from other nuclear components on a step sucrose gradient followed by a continuous Percoll-sucrose gradient. This gra-

Table I. Specific Activities of SPB-containing Fractions from Various Stages of the SPB Enrichment Procedure

Sample*	Volume	Total number of SPBs‡	Total protein	Specific activity
	ml		*mg*	*SPBs/μg protein*
S. uvarum NCYC74 spheroplasts	320	4.0×10^{11}	5800	6.9×10^{4}
Nuclei (2.1/2.3 M sucrose fraction)	113	3.4×10^{11}	500	6.8×10^{5}
Crude SPBs (2/2.25 M sucrose fraction)	20	2.4×10^{11}	38	6.3×10^{6}
Percoll enriched SPB fraction	0.38	1.8×10^{11}	4.4	4.1×10^{7}

* Fractions collected as described in Materials and Methods.
‡ SPB numbers for spheroplasts were obtained by assuming 1.5 SPBs/spheroplast (Baum et al., 1988) in a count of 2.7×10^{11} spheroplasts.

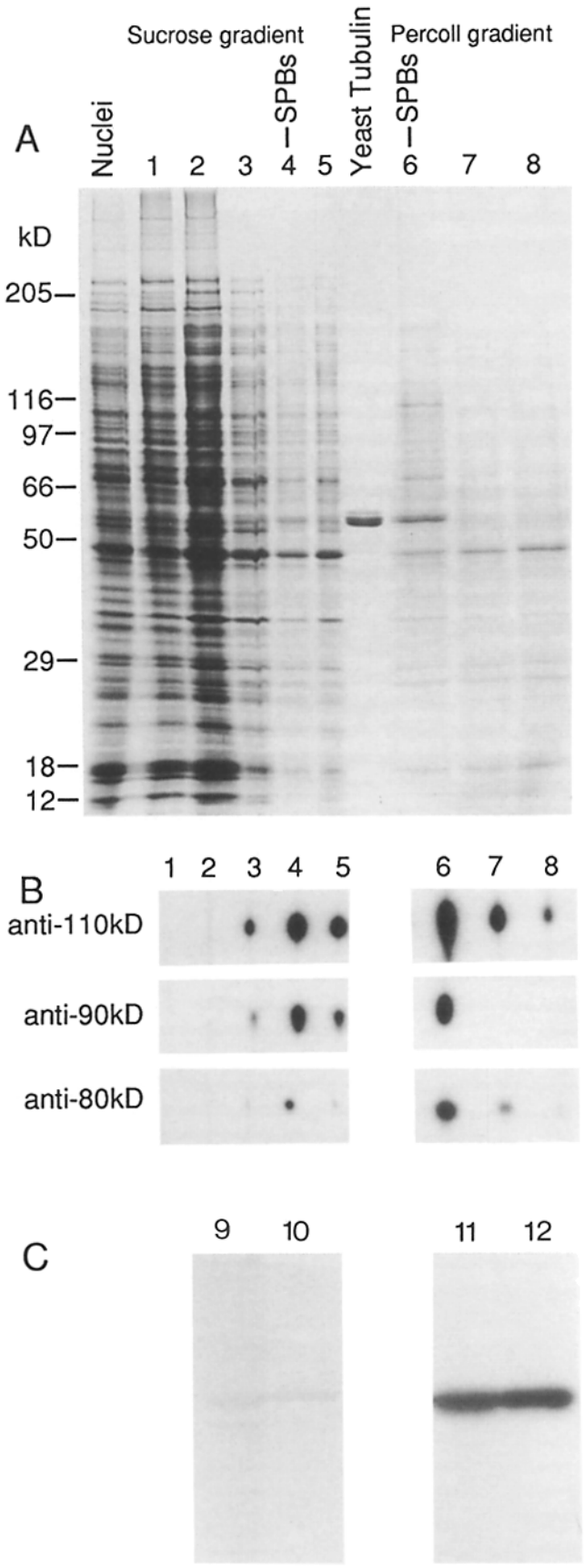

Figure 2. (*A*) SDS gel electrophoresis of fractions during SPB enrichment showing enrichment in SPBs of a band comigrating with yeast tubulin. Gradient fractions were unloaded in approximately equal volumes and equal volumes were loaded onto each lane. In the sucrose gradient, lane *1* is the sample layer, lane *2* is the layer between this and the 1.75 M sucrose layer, lane *3* is the 1.75–2.00 M sucrose layer, lane *4* is the 2.00–2.25 M layer and lane *5* the 2.25–2.50 M sucrose layer. Lanes *6–8* are from the Percoll gradient as explained in Materials and Methods. Lanes *4* and *6* contained 70% of the total SPBs loaded onto each gradient. (*B*) Immunoblots of lanes *1–8* showing that the maximum signal coincides with the maximum concentration of SPBs. (*C*) Lanes *9* and *10* show a Ponseau S stained blot with approximately equal loadings of the band comigrating with yeast tubulin in Percoll SPBs (lane *9*) and pure yeast tubulin (lane *10*). When reacted with the antitubulin mAb YOL1/34 (Kilmartin et al., 1982), approximately equal signals were obtained (lanes *11* and *12*), showing that the comigrating band in Percoll SPBs is mainly tubulin.

dient used an unusual combination of low Percoll concentration and high sucrose concentration. When such a combination was spun at high speed it formed a continuous density gradient with a sigmoid profile. With the appropriate combination of sucrose and Percoll we found that SPBs remained in the shallow part of this gradient and separated from contaminating material that had only slightly different buoyant densities.

Examination of thin sections of the final enriched SPB fraction by EM (Fig. 1) showed that it contained morphologically intact SPBs, similar to those seen in native yeast cells (Moens and Rapport, 1971), together with attached and some detached microtubules. This final fraction was pelleted twice by centrifugation to concentrate it, so the longer microtubules had suffered some shearing damage. In contrast, material from the cruder 2.00/2.25-M sucrose gradient fraction (see Materials and Methods) contained SPBs of similar morphology but with a many intact spindles with both apparently pole to pole and discontinuous microtubules and SPBs at either end (see Fig. 6). A significant proportion of the SPBs were paired side by side, as has been observed by others in their SPB isolates (Hyams and Borisy, 1978; Byers et al., 1978). Many of these pairs were clearly not joined by microtubules, and in some examples two faint parallel strands could be seen joining the central plaques of the paired SPBs. In negatively stained samples of DEAE-dextran treated SPBs (see Fig. 8 *c*) the strands were particularly clear. These paired SPBs presumably correspond to the paired SPBs connected by a bridge structure seen in cells during the first part of bud formation (Byers and Goetsch, 1975). In thin sections it was noticeable that the SPBs had a pronounced curvature, being concave on the nuclear and convex on the cytoplasmic side.

The SPB-containing fractions were concentrated and enriched enough to begin structural studies on native unstained SPBs embedded in vitreous ice using cryoelectron microscopy (in collaboration with C. Akey). The images obtained by this method suggested an SPB morphology similar to that deduced from negatively stained and thin sectioned fixed SPBs, though in the case of vitreous material additional structures could be seen, particularly in the region of the intermediate line (Fig. 1, *inset*; for nomenclature see Fig. 11).

The SPBs from all fractions were competent to nucleate microtubule growth when exogenous brain tubulin was added; indeed, this was the basis for our assay for SPBs.

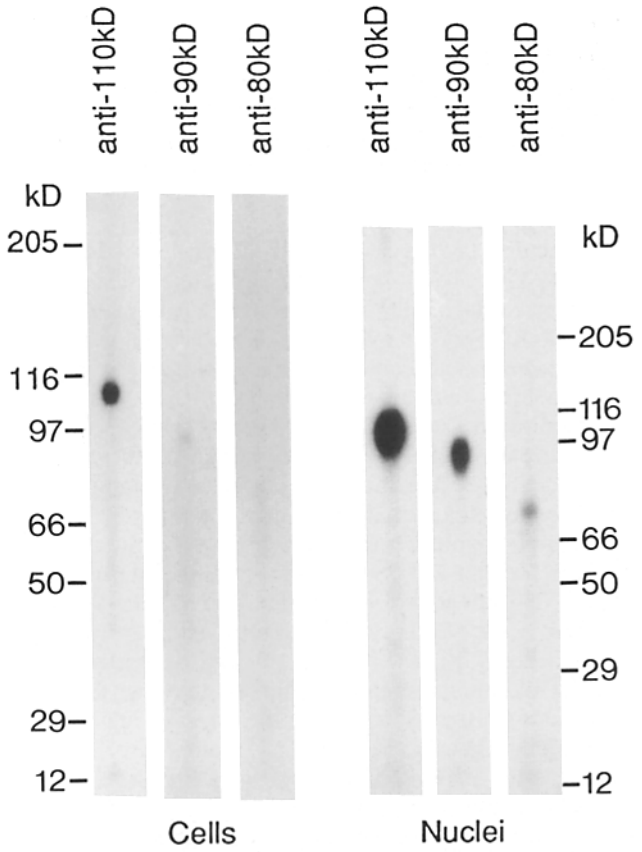

Figure 3. Immunoblots of yeast whole cell extracts and nuclei reacted with the anti-110-kD mAb 45D10, the anti-90-kD mAb 21D9 and the anti-80-kD mAb 34E12.

Since the inclusion of DMSO caused retention of the endogenous yeast microtubules attached to the SPBs, the brain microtubules presumably grow from these. The mean number of microtubules nucleated per SPB was 26 ± 5, as compared with 9 and 28 (Hyams and Borisy, 1978) and 18 (Byers et al., 1978) for other diploid SPB preparations. All the microtubules nucleated by the enriched SPBs grew from the nuclear SPB face, which was found also by Hyams and Borisy (1978). During cell lysis the capacity of the cytoplasmic face of the SPB to nucleate microtubule polymerization was irreversibly lost.

Specific activity measurements (see Table I) showed that the final fraction of SPBs from the Percoll gradient was enriched ~600-fold over whole yeast cells, with a total yield of ~45%. These numbers should be considered approximate because of inaccuracies in counting SPBs and possible separation of paired SPBs during enrichment giving a higher than expected yield. SDS PAGE of the final SPB fraction confirmed this level of purity (Fig. 2 *A*, lane *6*) by showing a substantial enrichment for yeast tubulin. Tubulin is a minor protein in yeast (Kilmartin, 1981), but in the final SPB fraction the protein comigrating with the yeast tubulin standard was the major protein, accounting for 20% of the total. The identification of this band as mainly tubulin was confirmed by comparing immunoblots of the SPB fraction and purified yeast tubulin stained with antitubulin. When similar amounts of the two bands were loaded, the signal from the tubulin position in the final SPB fraction was nearly as intense as the signal from pure tubulin (Fig. 2 *C*). Two-dimensional gel electrophoresis also showed the characteristic pattern of α and β tubulin spots (Kilmartin, 1981) but since a substantial proportion of the proteins did not run in the isoelectric focusing dimension (data not shown), this was a less useful method for establishing the identity of this band. It was also noticed that a number of minor proteins, particularly a band at 110 kD, copurified with yeast tubulin during the fractionation procedure.

mAbs

The enriched SPB fractions were used to produce mAbs against spindle and SPB components. The primary screen was by immunofluorescence of isolated SPBs from the 2.00/2.25-M sucrose gradient fraction. A large proportion of these SPBs were present as doublets tethered to each other either by microtubules or by a short strand of unstructured material as others have previously found (Hyams and Borisy, 1978; Byers et al., 1978). Thus a positive result was easily recognizable as a paired immunofluorescent dot staining pattern and readily distinguishable from a random dot staining pattern caused by, for example, sticky aggregated antibodies. Three fusions gave ~15,000 hybridomas and 60 positives by the primary screen. 38 of these were stable enough to be cloned by limiting dilution. 34 of the stable lines gave clear SPB or spindle staining in spheroplasts and 14 of these (all IgGs) gave signals on immunoblots (Table II), allowing us to identify at least three different SPB and spindle components. A fourth component of apparent molecular mass 40 kD was also identified during this series of fusions, and the mAb reacting with this appeared to stain the mitotic spindle. But this antibody was an IgM and has proven difficult to characterize, so it is not included here.

Immunoblots

The 14 mAbs which gave a signal on immunoblots of enriched SPBs could be grouped into three classes (Table II) recognizing components of apparent molecular masses 110, 90, and 80 kD. All but one of the monoclonal antibodies recognized a single band on immunoblots, indicating that the components are different and distinct rather than proteolytic fragments of one or two other proteins. The exception, 45H10, cross-reacted with one other band on blots of whole

Table II. mAbs Obtained against Yeast SPB and Spindle Components

mAb		Antigen	Immunofluorescent staining
		kD	
3D2	36G6	110	SPB
23G3	45D10		
24A8	45H10		
30G11	48G4		
35A11			
21D9	35B5	90	SPB
33C9	48B6		
34E12		80	SPB and particulate staining of short spindles

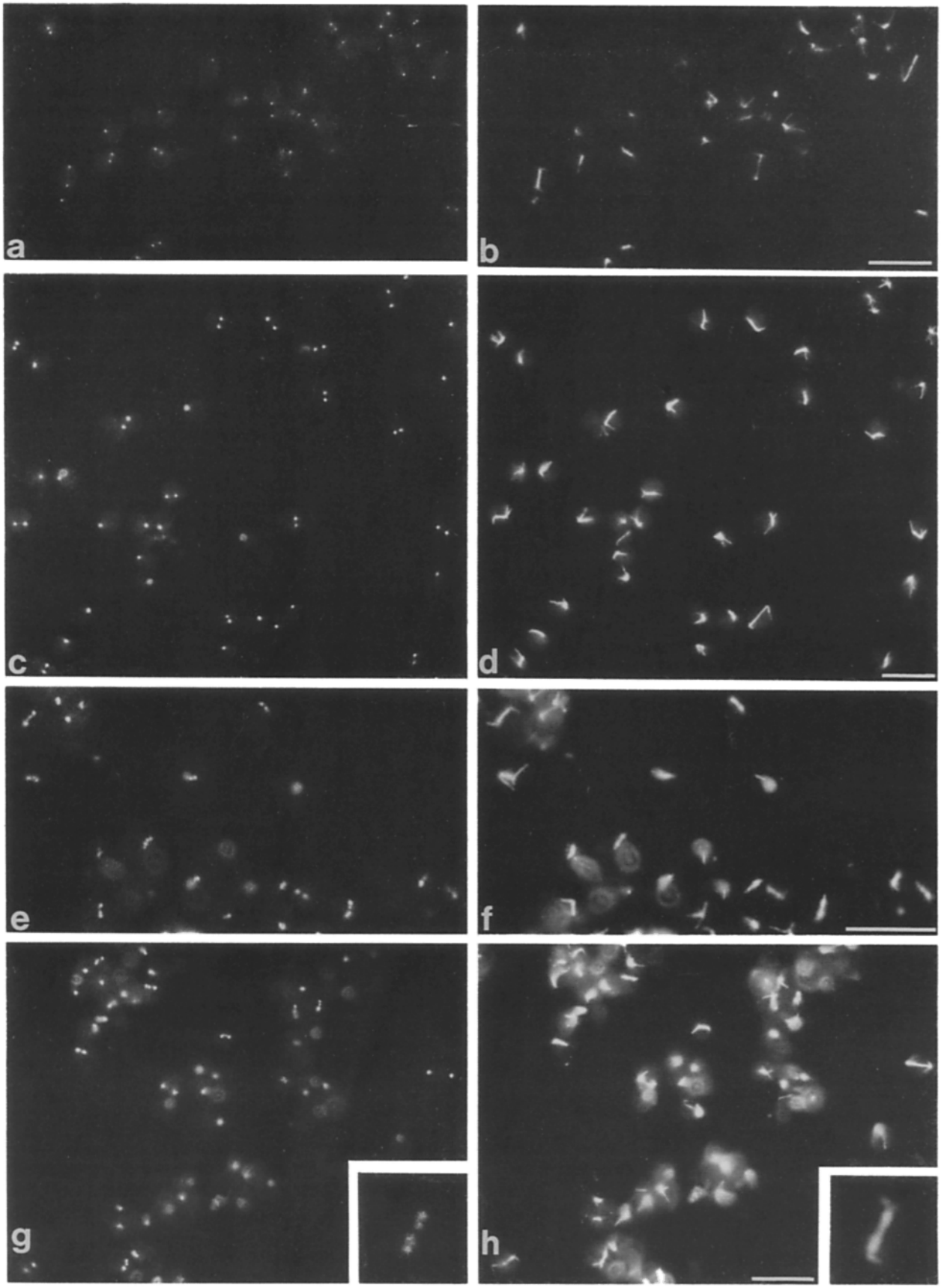

Figure 4. Double label immunofluorescence of spheroplasts labeled with mAbs (*left*) and rabbit antitubulin (*right*). (*a* and *c*) Spheroplasts labeled with the anti-110-kD mAb, 3D2 and the anti-90-kD mAb 21D9, respectively. The dot staining obtained with the mAbs coincides with the SPB region, the junction between the thick bundle of spindle microtubules and the thinner sometimes multiple bundles of cytoplasmic microtubules. (*e* and *g*) Two fields of spheroplasts labeled with the anti-80-kD mAb 34E12. (*Inset*) An enlargement of an individual spheroplast. The staining pattern is similar to *a* and *c* but short spindles often have additional dots. Bar, 10 μm.

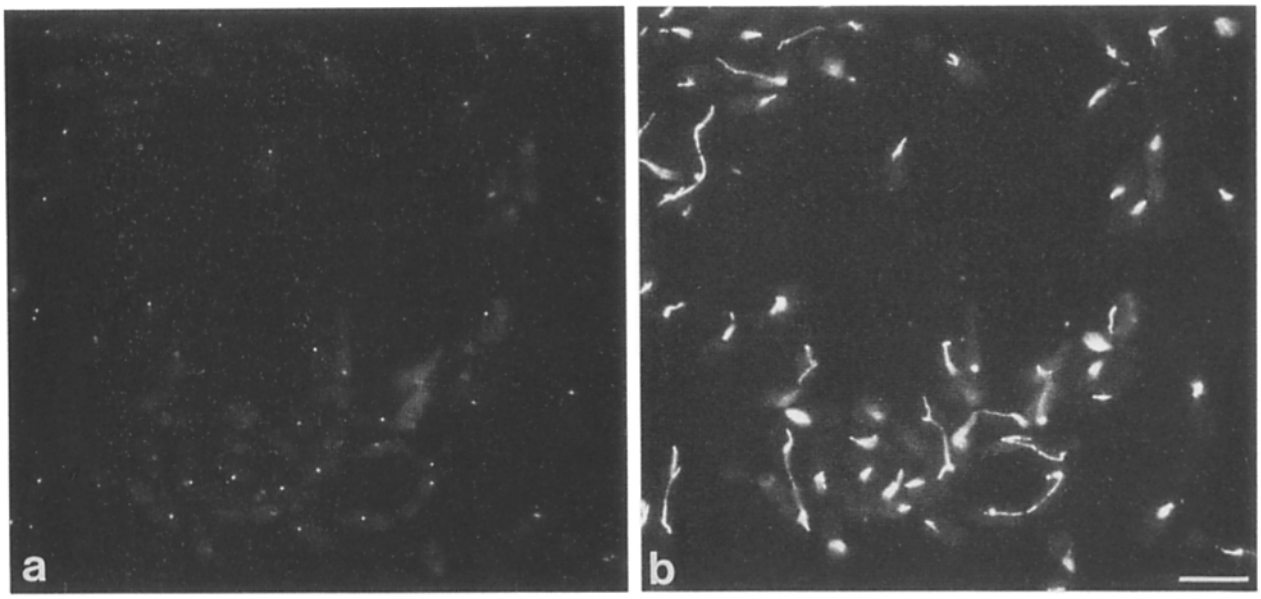

Figure 5. Double-label immunofluorescence of cells fixed in formaldehyde for 15 min and labeled with the anti-90-kD mAb 21D9 (*a*) and rabbit antitubulin (*b*). The dots stained by 21D9 coincide with the forked or bent junction between nuclear and cytoplasmic microtubules. Bar, 10 μm.

cell extracts. The signal from the anti-110-kD mAbs migrates close to the Coomassie blue–stained 110-kD band apparently coenriching with SPBs (Fig. 2 *A*). In whole cell immunoblots (Fig. 3) only 45D10 and 45H10 of the anti-110-kD antibodies and 21D9 of the anti-90-kD antibodies gave detectable staining. The staining given by 21D9 was very weak, whereas the stronger staining given by 45D10 is probably a result of the very high titer of this supernatant, since detectable staining of the 110-kD band was still obtained on immunoblots of enriched SPBs with 1 in 10^6 dilutions. There was no detectable staining of whole cell immunoblots with the anti-80-kD mAb 34E12. All the antibodies stained immunoblots of yeast nuclei (Fig. 3) reacting with a single band of the appropriate size. We were not able to establish whether the antigens were exclusively nuclear by showing their absence in post-nuclear supernatants for two reasons. Firstly the immunoblot signal was too weak for the 90- and 80-kD components. Secondly, although the signal was strong enough using the antibody 45D10 for the 110-kD component and appeared absent in postnuclear supernatants, a definite conclusion could not be reached because control experiments showed that the PVP present interfered strongly with the blotting causing too great a variability in the signals. Attempts to remove the PVP were unsuccessful.

On both the sucrose step gradient and the Percoll continuous gradient the maximum reactivity of the antibody with its antigen was always coincident with the peak of SPB activity (Fig. 2 *B*). Only the part of the immunoblot containing the particular antigen is shown because like the immunoblots of nuclei (Fig. 3), no other bands were detectably stained.

The anti-110-kD mAbs are probably all against the same 110-kD component since they all react with the same λgt11 phage isolated by screening a λgt11 genomic DNA expression library (Snyder and Davis, 1988); they also define several different epitopes within the 110-kD component as judged by their reactivity with phage containing partial inserts (Kilmartin, unpublished results). We have not yet established whether the anti-90-kD mAbs are against a single 90-kD component, though the staining intensity was much greater with combinations of these antibodies compared with similar dilutions of single antibodies, suggesting different epitopes.

Immunofluorescence Microscopy

During the initial characterization of the mAbs using isolated SPBs, it was found that covalently cross-linking fixatives such as formaldehyde or glutaraldehyde decreased their affinity to varying degrees. This decrease in affinity was such that under normal fixation conditions for yeast cells (60–90 min of formaldehyde fixation for optimum preservation of microtubules [Kilmartin and Adams, 1984; Adams and Pringle, 1984]) no signal was given by the antibodies. This problem was circumvented by a different fixation procedure not requiring formaldehyde, but using instead methanol and acetone to fix cultured spheroplasts. The mAbs against both the 110- and 90-kD components gave near identical immunofluorescence staining patterns in yeast spheroplasts, labeling a dot or pair of dots in each cell. In double labeling experiments with antitubulin (Fig. 4, *a*, *b*, *c*, and *d*), the position of each dot in cells with two dots was exactly coincident with the junction between the thin bundle of cytoplasmic microtubules and the thick bundle of nuclear microtubules where the SPB is present (Byers, 1981*b*). In cells with one dot, the staining was coincident with the center of the radiating microtubules, again the expected position of the SPB (Byers, 1981*b*). All cells give this one or two dot staining pattern with antibodies for both the 110- and the 90-kD component, suggesting the presence of these components in the SPB throughout the cell cycle.

The mAb against the 80-kD component gave a staining pattern superficially similar to that obtained from the anti-

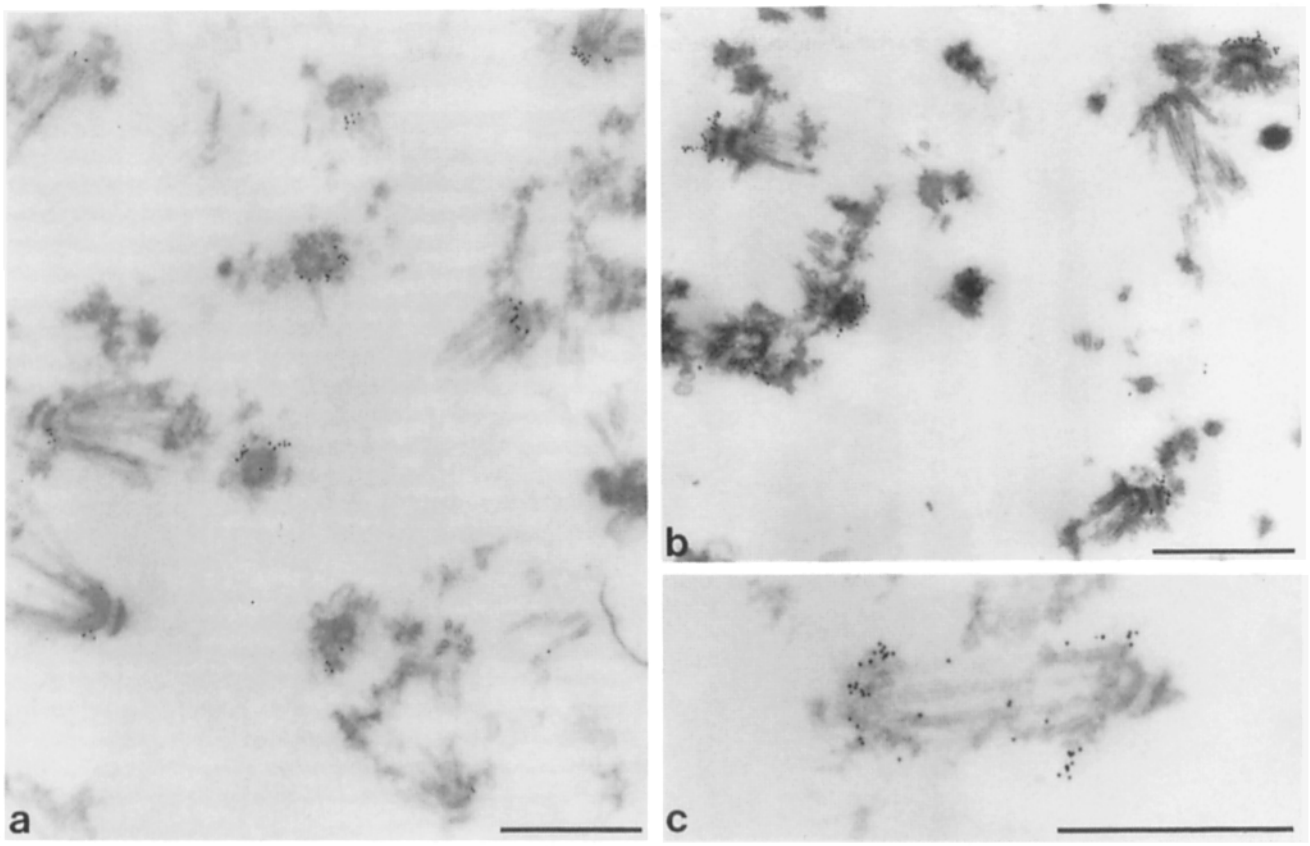

Figure 6. Immunoelectron microscopy of intact SPBs and spindles labeled with (*a*), a mixture of anti-110-kD mAbs; (*b*) the anti-90-kD mAb 35B5; and (*c*), the anti-80-kD mAb 34E12. Bar, 0.5 μm.

90-kD or anti-110-kD mAbs but which differed in a number of details (Fig. 4, *e*, *f*, *g*, and *h*). The dot staining appeared slightly more diffuse and was not located at the junction of the cytoplasmic and nuclear microtubules but rather was slightly to the spindle side of that junction. This can be seen in cells with spindles by measuring the distance between the two end dots of anti-80-kD staining and the spindle length represented by the distance between the two junctions of the thin cytoplasmic microtubule bundles and the thick nuclear microtubule bundles. In cells stained with the anti-80-kD antibody the distance between the dots was consistently less than the spindle length in comparison to the equivalent distances with the anti-90-kD and anti-110-kD antibodies. In addition, cells stained with the anti-80-kD antibody showed particulate staining of most short spindles, which appeared to be absent in long spindles. Some short spindles had only one area of additional spindle staining associated with the midpoint of the spindle or displaced slightly to one side. In other rarer views there were two areas on the spindle axis placed symmetrically either side of the spindle midpoint (Fig. 4 *g*, *inset*).

The anti-90-kD antibodies were less susceptible to formaldehyde fixation than the anti-110-kD and anti-80-kD antibodies, and thus we were able to stain yeast cells briefly (15–60 min) fixed with formaldehyde. Fig. 5 shows double labeling of such cells with 21D9 and antitubulin. As in the sphero-

Table III. Distribution of Gold Particles on Immunostained SPBs, Spindles, and Extracted SPBs

SPB type	Antigen	Number of SPBs analyzed	Total gold particles counted	Location of gold particles					Gold particles per SPB in major location
				Nuclear face of SPB	Cytoplasmic face of SPB	Side of SPB	Spindle microtubules	Elsewhere	
	kD			%	%	%	%	%	
Untreated	110	208	726	82.2	0.3	1.1	0	16.4	2.9
	90	411	3049	10.1	79.7	4.7	0	5.5	5.9
	80	202	1776	0	0	0	87.2	12.8	7.7
DEAE extracted	110	112	254	85.0	0.4	2.8	0	11.8	1.9
	90	220	2465	40.0	47.7	3.3	0	9.0	4.5 & 5.3
Heparin extracted	110	214	1306	90.9	0.2	1.4	0	7.5	5.6

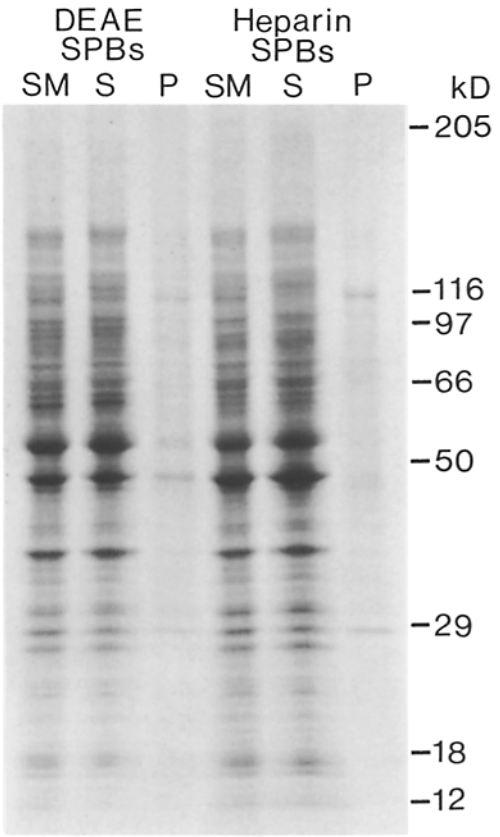

Figure 7. SDS gel electrophoresis of DEAE-dextran and heparin extracted SPBs. *SM*, starting material; *S*, supernatant after extraction; *P*, material from the 2.0–2.5-M sucrose gradient interface.

plasts (Fig. 4 *c*) each cell has one or two dots in the position expected for the SPB, that is coincident with the junction between cytoplasmic and nuclear microtubules, where the microtubules are abruptly bent or forked.

Immunoelectron Microscopy

The mAbs were also used to localize the three antigens by immunoelectron microscopy on SPBs from the 2.00/2.25-M sucrose gradient fraction. Due to the fixation sensitivity of the antigens and the poor ultrastructural preservation of methanol and acetone fixed specimens, immunostaining was performed on native unfixed SPBs and their associated spindle microtubules. The results confirmed the position of the antigens as seen by immunofluorescence, though clear differences in the positions of the staining within the SPB were seen for the different antibodies. The 110-kD component was localized to the nuclear side of the SPB (Fig. 6 *a* and Table III). In contrast, the 90-kD component was localized mainly to the cytoplasmic face of the SPB, though a small proportion of the gold particles (~10%) were also found on the nuclear face of the SPB (Fig. 6 *b* and Table III). Two of the anti-90-kD antibodies were tested (21D9 and 35B5), and both gave similar staining. The 80-kD component was associated with the spindle microtubules, in particular the region close to the SPB (Fig. 6 *c* and Table III). In comparative counts of gold particles along intact spindles the gold particle density close to the SPB was twice as high with the anti-80-kD antibody 34E12 compared with a mAb against tubulin, YL1/2 (Kilmartin et al., 1982).

Chemical Extraction of the SPBs

Because the SPB is a compact structure and the reactivity of antigens in it may be blocked by their close association with other SPB components, chemical extractions were developed to produce subparticles of the SPB that might remove components causing steric blocking and thus increase the reactivity of the antibodies. It has been reported that soluble charged polymers are very effective in subnuclear fractionation (Bornens, 1973), and indeed both DEAE-dextran and heparin were found to extract the SPBs to varying degrees. These produced SPB residues more highly enriched and with less complex SDS-PAGE patterns than any of the intact SPB fractions (Fig. 7): for example, compare the difference in Coomassie Blue staining in the tubulin region between the Percoll SPBs (Fig. 2 *A*, lane *6*) and the chemically extracted SPBs (lanes *P*, Fig. 7).

DEAE-dextran extraction of SPBs (DEAE-SPBs) removed all the microtubules but the gross morphology of the SPB appeared intact including the cytoplasmic outer plaque, the central plaque, an inner plaque and the unstructured material holding pairs of SPBs together (Fig. 8, *a*, *b*, and *c*; for nomenclature see Discussion and Fig. 11). The central and inner plaques also appeared to be connected by filaments (Fig. 8 *b*). Immunoblots showed that DEAE-SPBs retained both the 90- and 110-kD components but lost the 80-kD component and tubulin (Fig. 9).

EM showed that heparin treatment produced SPB residues (heparin-SPBs) retaining the central plaque and some of the intermediate line but lacking microtubules, the inner plaque, most of the cytoplasmic outer plaque and the unstructured material holding pairs of SPBs together (Fig. 8 *d*). Immunoblots showed that only the 110-kD antigen was retained in heparin-SPBs (Fig. 9).

These chemical extractions gave independent evidence for the localization of the antigens in the SPB. Thus, the microtubule-associated 80-kD component was removed with the tubulin, and the 90-kD component was removed when the cytoplasmic outer plaque and nuclear inner plaque were largely extracted with heparin. The 110-kD component was retained with the central plaque in both the heparin and DEAE-SPBs.

Immunoelectron Microscopy of the Chemically Treated SPBs

In intact SPBs, the anti-90-kD mAbs mainly labeled the cytoplasmic face of the SPB, although there was lower but significant labeling of the nuclear side: compare the ratios of cytoplasmic to nuclear labeling with the anti-110- and anti-90-kD antibodies in Table III. It was possible that the lower degree of labeling of the nuclear side was due to steric blocking by the spindle microtubules. When they were removed by DEAE-dextran extraction, the degree of labeling of the nuclear side of the SPB with the anti-90-kD antibodies was dramatically increased, while the degree or position of the staining on the cytoplasmic face of the outer plaque was unaltered (Fig. 10 *a* and Table III). Similarly the position and degree of labeling on the nuclear concave side of the SPB with the anti-110-kD antibodies remained unaltered in

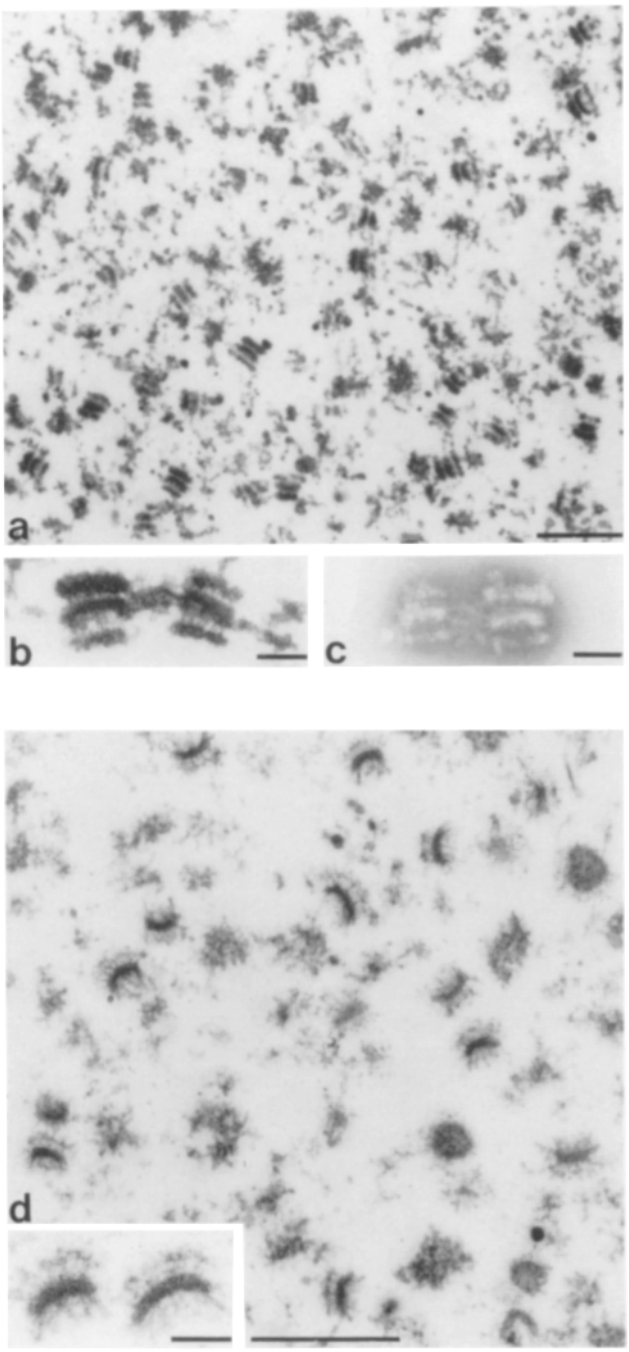

Figure 8. (*a*) Thin section of a pellet of DEAE-dextran extracted SPBs. (*b* and *c*) Thin section (*b*) and negative stain (*c*) of a pair of DEAE-dextran extracted SPBs joined by a bridge structure. (*d*) Thin section of a pellet of heparin extracted SPBs. (*Inset*) enlargement of two heparin-extracted SPBs. Bars: (*a* and *d*) 0.5 μm; (*b* and *c*) 0.1 μm; (*inset*) 0.1 μm.

DEAE-SPBs (Fig. 10 *b* and Table III). However, when the inner nuclear plaque was removed by heparin extraction, the degree of anti-110-kD labeling increased (Fig. 10 *c* and Table III), presumably again due to removal of steric blocking. The positions of the 90- and 110-kD antigens on the nuclear side of the SPB appeared to be significantly different. In DEAE-SPBs labeled with the anti-90-kD antibody 35B5 the gold

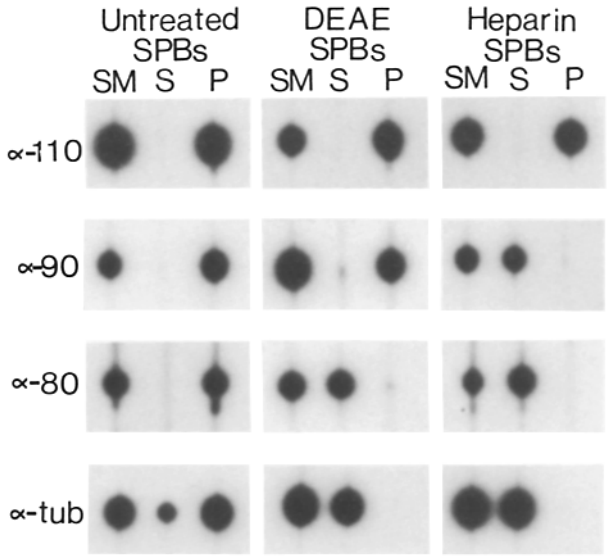

Figure 9. Immunoblot of fractions from the DEAE-dextran and heparin extractions of SPBs showing that the 80-kD component and tubulin are removed in DEAE-dextran and heparin extracted SPBs, the 110-kD component remains in both, and the 90-kD component is removed by heparin but not by DEAE-dextran extraction. *SM*, starting material; *S*, supernatant after extraction; *P*, material from the 2.0–2.5-M sucrose gradient interface. *α-110*, blots reacted with the anti-110-kD mAb 3D2; *α-90*, blots reacted with the anti-90-kD mAb 35B5; *α-80*, blots reacted with the anti-80-kD mAb 34E12; and *α-tub*, blots reacted with the anti-tubulin mAb YOLI/34.

particles were 50 ± 20 nm (20 SPBs, a total of 156 gold particles measured) from the nuclear face of the central plaque. The equivalent distance for the anti-110-kD antibody 3D2 was 24 ± 15 nm (20 SPBs, a total of 157 gold particles measured) for labeled heparin SPBs and 23 ± 9 nm (20 SPBs, a total of 49 gold particles measured) for DEAE-SPBs. These differences in labeling position on the nuclear side of the SPB suggest that the 110-kD antigen is close to the nuclear side of the central plaque whilst the 90-kD antigen is close to the inner plaque.

Discussion

This paper describes an enrichment procedure for producing SPBs and associated spindle microtubules that retain most of the morphological features seen in thin sections of intact cells (Moens and Rapport, 1971). The SPBs were produced in sufficient quantities to raise mAbs that were used to identify three components and to localize them to specific parts of the SPB and spindle (see Fig. 11). Although the bulk of the SPB is clearly intact, it is possible that SPB components involved in some other functions may have been lost. In common with other SPB isolation methods (Byers et al., 1978; Hyams and Borisy, 1978; Kilmartin and Fogg, 1982), cytoplasmic microtubules are lost together with the ability of the cytoplasmic face of the SPBs to nucleate microtubules, due presumably to removal or denaturation of cytoplasmic microtubule nucleating proteins. Dispersal of the nuclear membranes with detergents could also have removed proteins that anchor the SPBs into the nuclear envelope, as well as some components of the bridge structure connecting

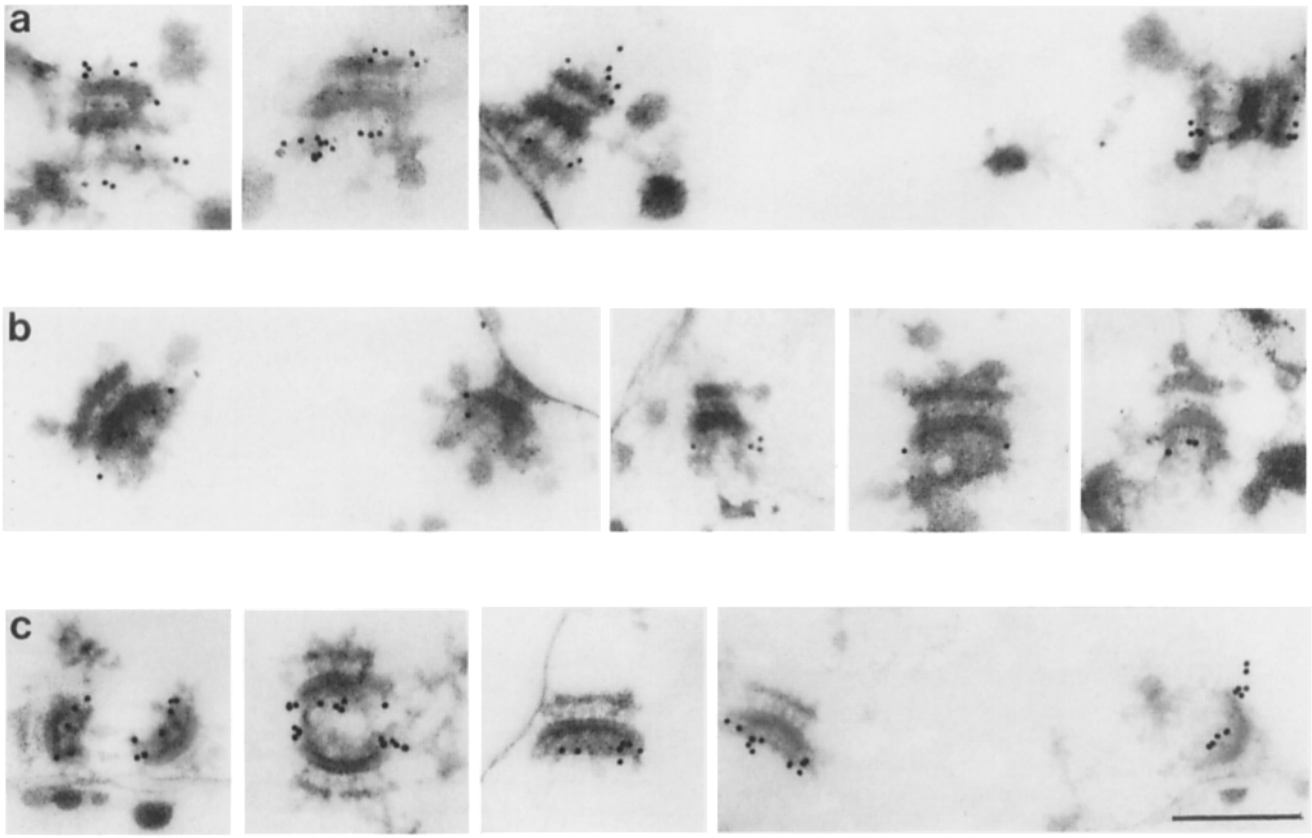

Figure 10. Immunoelectron microscopy of DEAE-dextran (*a* and *b*) and heparin (*c*) extracted SPBs reacted with the anti-90-kD mAb 35B5 (*a*) and the anti-110-kD mAb 3D2 (*b* and *c*). Bar, 0.2 μm.

paired SPBs (Byers and Goetsch, 1975). However, it is likely that the microtubule attachment or nucleation sites are still present on the nuclear side of the enriched SPBs because the endogenous nuclear microtubules remain attached.

Morphology of the Chemically Extracted SPBs

The chemical extraction procedures revealed some further structural aspects of the SPBs. With DEAE-dextran the extracted SPBs appeared morphologically intact. However, a previously obscured part of the nuclear face of the SPB, called here the inner plaque (Fig. 11), was now revealed. This may be the same as the "dense zone" or "inner zone" present on the nuclear face of meiotic SPBs (Moens and Rapport, 1971; Peterson et al., 1972); and although not obvious in mitotic cells (Moens and Rapport, 1971), there does seem to be some structure at this position, as the spindle microtubules appear to end some distance from the central plaque in the approximate position of the inner plaque (Moens and Rapport, 1971; Byers and Goetsch, 1975; King et al., 1982). The inner plaque is less visible than the other plaques probably because it is somewhat less osmophilic (Fig. 8 *a*) and obscured by the proximal ends of the spindle microtubules. It is possible to see this inner plaque in our enriched intact SPB fractions, though it is clearest in the vitreous ice samples (Fig. 1, *inset*). However, it may be that the inner plaque is more prominent in our SPB preparations because our spheroplasts may have begun to enter meiosis due to starvation during cell wall removal, which, to economize on enzymes, was prolonged for up to 3 h. There is some evidence, especially in preparations of the DEAE-SPBs, that the inner plaque is connected to the central plaque by filaments (Fig. 8 *b*).

SPBs extracted with heparin had a completely different appearance: the inner plaque and most of the cytoplasmic outer plaque were removed while the central plaque and part of the intermediate line were retained, and the characteristic curved morphology of the central plaque was also retained with at least part of the intermediate line present on the convex side.

Purity and Protein Composition of the SPB Fractions

The purity of the SPBs prepared by our enrichment procedure is difficult to estimate because we do not know the specific activity of pure SPBs. A tentative estimate of ~10% purity is based on the abundance of SPBs in thin sections of the final pellet (Fig. 1) and the relative proportion of tubulin (20%) in this final fraction. If it is assumed that the protein density in microtubules and SPBs is similar, then since the bundle of microtubules attached to the SPB has the same diameter as the SPB and the total length of the SPB is about half the average length of its attached microtubules, its protein mass would be half the microtubule mass, that is 10% of the final fraction. This is similar to the level of purity obtained in centrosome enrichment procedures (Mitchison and Kirschner, 1984). The probability that our SPBs are ~10% pure explains why both the 90- and 80-kD components are not seen as Coomassie blue–stained bands in the final Percoll

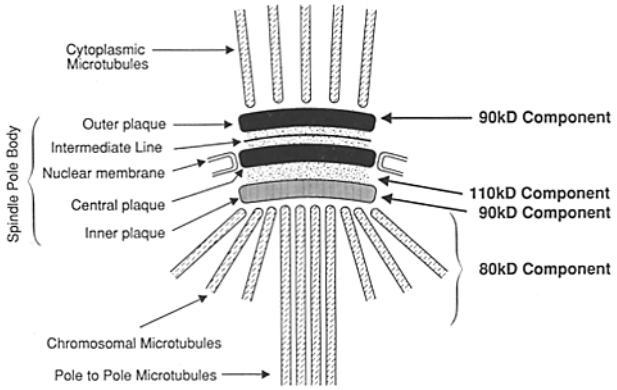

Figure 11. Summary diagram of the localizations of the SPB and spindle components. The nomenclature used is a combination of that used by others (Moens and Rapport, 1971; Peterson et al., 1972; Byers and Goetsch, 1975). The outer plaque is the same as the outer plaque of both Moens and Rapport (1971) and Byers and Goetsch (1975), and the outer zone of Peterson et al. (1972). The central plaque is the same as the inner plaque of Moens and Rapport (1971), the spindle plaque of Byers and Goetsch (1975) and the central zone of Peterson et al. (1972). The inner plaque is the same as the dense zone of Moens and Rapport (1971) and the inner zone of Peterson et al. (1972).

fraction (Fig. 2 *A*, lane *6*). Although present in multiple copies as indicated by the immunoelectron microscopy (see below) they would still be a small proportion of the total SPB mass and would be swamped by the 70% of impurities present. The heparin and DEAE-SPBs are probably more enriched than the Percoll SPBs as judged by the comparative simplicity of their SDS gel patterns (Fig. 7). The immunoblots show clearly that the 90-kD component is present in the DEAE-SPBs and absent in the heparin SPBs. A careful examination of the SDS gel patterns of the two SPB preparations in the 90-kD region does show a faint extra Coomassie blue–stained band in the DEAE-SPB fraction. If this is indeed the 90-kD component, it is clearly present in much lower amounts than the band at 110 kD, assuming both bind Coomassie blue equally. The question then arises as to why there is a difference in the relative amounts of the two components, particularly as the 90-kD component is apparently present in two parts of the SPB, whereas the 110-kD component is present in only one part. It may be that the 110-kD component recognized by our mAbs is a small proportion of the 110-kD Coomassie blue–stained band or the 110-kD component is present in many more copies per SPB.

Properties of the mAbs

Three different components associated with the SPB or spindle have been identified by the production of specific mAbs. Each set of antibodies specifically stained SPBs or spindles in whole yeast spheroplasts by immunofluorescence and stained specific structures in isolated SPBs and spindles by immunoelectron microscopy. During nuclear fractionation the SPBs (as assayed by their biological activity) also cofractionated with the three different components as detected by immunoblotting, indicating that the components stained by immunofluorescence and immunoelectron microscopy corresponded to the components identified by the antibodies on immunoblots. Furthermore, the dissection of the spindles and SPBs by chemical treatments confirmed and extended the localization of the three components to particular parts of the SPB and spindle in the intact structure. Thus, the positions of each of the three components have been assigned on the basis of two independent methods: immunolocalization by electron microscopy in intact SPBs and spindles, and chemical dissection of the spindle apparatus, following the components by immunoblotting and immunolocalization.

The three SPB and spindle components we have identified do not appear to be related to the other possible SPB components mentioned in the Introduction. *CDC31*, *SPA1*, and *KAR1* have considerably lower molecular weights (Baum et al., 1986; Snyder and Davis, 1988; Rose and Fink, 1987), and the immunofluorescent staining pattern given by the *NSP1* gene product is quite different from any of the staining patterns given by our mAbs (Hurt, 1988).

One difficulty encountered in identifying the antigens by immunofluorescence on whole yeast cells was their sensitivity to formaldehyde and other covalent cross-linking fixatives. The degree of sensitivity varied both with the particular component stained and the individual antibody used, and the reasons for this are probably complex. That the epitopes recognized by the antibodies were inherently sensitive to covalent modifications could be shown by the decreased titer of the antibodies on blots pretreated with formaldehyde. The tightly packed structure of the SPB and steric blocking by both cytoplasmic and nuclear microtubules and the nuclear envelope might also have restricted access of antibodies against its components; the observed increase in immunostaining of both the 110- and 90-kD components on the selective removal of adjacent material supports this (Table III), and similar access problems have been demonstrated in another densely packed structure, the mammalian midbody (Saxton et al., 1984). Yeast, moreover, has a very compact cytoplasmic and nuclear structure (Peterson and Ris, 1976) and it has been shown that an increase in covalent fixation of yeast cells causes a decrease in the ability of antibodies to penetrate those cells (Kilmartin and Adams, 1984). This latter effect might explain why the immunofluorescence signals from the antigens were found to be uniformly less sensitive to formaldehyde treatment in isolated SPBs than they were in either spheroplasts or cells.

Because the noncovalent fixation procedures that were used did not chemically cross-link the samples, it cannot be excluded that some or all of the antigens recognized are ones which have become associated with the SPBs or spindles during or after fixation. However these antigens also cofractionate with SPBs during the enrichment procedure, so such an artefactual association would have to occur for the three distinct antigens during both fixation and cell fractionation. This seems an extremely unlikely possibility. Any remaining doubt as to the identity of these components should be resolved by cloning the corresponding genes and examining the phenotypes of mutations in these genes.

All the mAbs were used in an attempt to identify similar components in other organisms, but preliminary experiments suggested that the antibodies, at least in the concentrations present in cell supernatants, do not appear to cross-react widely.

The 110-kD Component

The mAbs against the 110-kD component recognized a single antigen present, like the others, throughout the cell cycle. It is present in multiple copies in each SPB since the gold particles were uniformly distributed over most of the labeled SPB layer once it had been exposed by chemical extraction (Fig. 10 *c*). The region of the SPB immunostained by the anti-110-kD monoclonals appeared to be a layer ~25 nm from the nuclear face of the central plaque (Fig. 6). Thus, this component could be a part either of the central plaque itself or of the region between the central and inner plaques. Immunoelectron microscopy on intact SPBs or DEAE-SPBs using antibodies against the 110-kD component gave a relatively low signal confined mainly to the side of the SPBs. The signal was increased considerably on removal of the inner plaque by heparin treatment, indicating that a proportion of the 110-kD component was hidden by this structure.

There are a number of features of the 110-kD component that suggest it might have a structural role in the SPB rather than being more directly involved in microtubule nucleation: (*a*) it is a component of the most central structures of the SPB, and the labeling is at least 30 nm from the nearest microtubule ends, the region where microtubule nucleation presumably occurs; (*b*) it is associated with the most persistent of the structures as defined by resistance to various chemical treatments; (*c*) it is only on one side of the SPB (assuming it has not been extracted accidentally from another part of the SPB during preparation), whereas microtubules in vivo are nucleated from both faces of the SPB.

The 90-kD Component

mAbs recognizing the 90-kD band on immunoblots decorated the cytoplasmic face of intact SPBs more or less uniformly by immunoelectron microscopy. There was a much smaller but significant degree of labeling of the nuclear side of the SPB (Table III). This increased dramatically when the SPBs were extracted with DEAE dextran, and microtubules that may have blocked reaction of the anti-90-kD antibodies with their antigen were quantitatively removed. The epitopes were present in multiple copies because they were uniformly distributed both on the outer cytoplasmic plaque of each SPB and in or on the inner nuclear plaque. The region immunostained on the nuclear side of the SPBs by the anti-90-kD antibodies was ~50 nm from the nuclear face of the central plaque. This distance was significantly different from that for the anti-110-kD antibodies (25 nm), suggesting that the two components are parts of different layers of the SPBs (Fig. 11).

Two lines of evidence point towards the 90-kD component being a possible part of the microtubule nucleating mechanism of the SPB: it is in multiple copies on each SPB, and has been localized to what appear to be the most extrinsic structures on both sides of the SPBs, close to where microtubules are nucleated. However, the inability of DEAE-SPBs to nucleate microtubule growth suggests that if the 90-kD component is in a "microtubule-nucleating element" then it is only a part of it, necessary but not sufficient for microtubule growth.

The 80-kD Component

The distribution of the 80-kD component is unlike the other two components. Immunoelectron microscopy shows it to be associated with the spindle microtubules, particularly those close to the SPB, whereas by immunofluorescence it is localized to discrete areas on the nuclear side of the SPB throughout the cell cycle, and is also found in regions that are on, slightly to one side, or distributed on either side of the midpoint of short spindles. Longer spindles, on the other hand, appear not to have such extra areas of staining. The 80-kD component thus shows cell cycle specificity with regard to its staining pattern. This staining pattern shows a striking resemblance to the "denser regions of chromatin" associated with the ends of discontinuous or possible chromosomal microtubules observed by Peterson and Ris (1976), which may correspond to the region containing kinetochores in yeast. At metaphase this material was distributed around the equatorial plane of the spindle or as a compact mass to one side of the spindle at the distal ends of the discontinuous microtubules. The pattern of midspindle staining with the anti-80-kD antibody 34E12 was very similar with a concentration of staining at or to one side of the spindle (Fig. 4, *c* and *d*). At anaphase, Peterson and Ris (1976) showed that the material could be resolved as two dense masses on either side of the equatorial plane of short spindles, which by late anaphase had become adjacent to the SPBs as the discontinuous microtubules shortened and the whole spindle lengthened. Similarly, in a subset of the shorter spindles the midspindle associated staining of the anti-80-kD antibody could be resolved into two areas on either side of the equatorial plane (Fig. 4, *inset*), which were apparently coincident with the two regions where the antitubulin staining of the spindle begins to thicken towards the spindle poles. These regions have been probably identified as the ends of the chromosomal microtubules (Kilmartin and Adams, 1984). In the longer anaphase spindles, only the poles were detectably stained. Thus the distribution at mitosis of the spindle-associated 80-kD component as seen by immunofluorescence is similar to that of the denser regions of chromatin presumably containing yeast kinetochores as seen by electron microscopy (Peterson and Ris, 1976). We tentatively propose that the epitope recognized by the anti-80-kD mAb 34E12 is a part of this dense material or of the yeast kinetochore, as well as of the polar regions of the spindle. The staining pattern observed with this antibody is also similar to that seen in diatom spindles when stained with MPM-2, an mAb against a family of phosphorylated mitosis-specific antigens (Davis et al., 1983; Wordeman et al., 1989) and a polyclonal antibody made against a 59-kD *Drosophila* microtubule binding protein which stains both centrosomes and kinetochores (Kellogg et al., 1989). The relationship, if any, of the 80-kD component to these proteins is not known at the moment, but like them, it may be associated with both the microtubules near the spindle poles and the kinetochores.

Because we have only one mAb (34E12) against the 80-kD component there is a possibility that the antibody is recognizing the same epitope in different antigens in the immunofluorescent staining and in the immunoblots. This seems unlikely since the signal from the immunoblots coenriches with the SPBs (Fig. 2 *B*).

Conclusion

In this paper, we characterize antigens other than tubulin that are almost certainly components of the yeast spindle appara-

tus. The techniques presented here are being refined and extended to identify more SPB and spindle components. Work is also in progress at the moment to clone and sequence the genes for the components already identified before carrying out genetic experiments to investigate their functions in vivo. A lethal phenotype might be expected if a gene encoding an SPB component was deleted; overexpression of this gene might produce aberrant SPBs or spindles. Such genetic analysis of the components identified here, together with the immunolocalization data, would also unequivocally establish their localization to the SPB or spindle.

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