

The Mammalian γ -Tubulin Complex Contains Homologues of the Yeast Spindle Pole Body Components Spc97p and Spc98p

Steven M. Murphy, Lenore Urbani, and Tim Stearns

Department of Biological Sciences, Stanford University, Stanford, California

Abstract. γ -Tubulin is a universal component of microtubule organizing centers where it is believed to play an important role in the nucleation of microtubule polymerization. γ -Tubulin also exists as part of a cytoplasmic complex whose size and complexity varies in different organisms. To investigate the composition of the cytoplasmic γ -tubulin complex in mammalian cells, cell lines stably expressing epitope-tagged versions of human γ -tubulin were made. The epitope-tagged γ -tubulins expressed in these cells localize to the centrosome and are incorporated into the cytoplasmic γ -tubulin complex. Immunoprecipitation of this complex identifies at least seven proteins, with calculated molecular weights of 48, 71, 76, 100, 101, 128, and 211 kD. We have identified the 100- and 101-kD components of the

γ -tubulin complex as homologues of the yeast spindle pole body proteins Spc97p and Spc98p, and named the corresponding human proteins hGCP2 and hGCP3. Sequence analysis revealed that these proteins are not only related to their respective homologues, but are also related to each other. GCP2 and GCP3 colocalize with γ -tubulin at the centrosome, cosediment with γ -tubulin in sucrose gradients, and coimmunoprecipitate with γ -tubulin, indicating that they are part of the γ -tubulin complex. The conservation of a complex involving γ -tubulin, GCP2, and GCP3 from yeast to mammals suggests that structurally diverse microtubule organizing centers such as the yeast spindle pole body and the animal centrosome share a common molecular mechanism for microtubule nucleation.

ORGANIZATION of the microtubule cytoskeleton occurs through a combination of site-specific nucleation by the centrosome and modulation of microtubule dynamics by interactions with microtubule motors and binding proteins. The centrosome nucleates the assembly of microtubules from soluble tubulin subunits, and maintains an attachment to the minus ends of many of the nucleated microtubules, resulting in a radial array centered at the centrosome. Microtubule motors and binding proteins modify this array, creating specialized structures, such as the mitotic spindle, from the generalized aster. Microtubules act as tracks upon which organelles and vesicles are moved, thus the organization of microtubules is essential to the higher order organization of the cytoplasm. In addition to α - and β -tubulin, which make up the microtubule polymer, there exists a third tubulin, γ -tubulin, that is localized to the centrosome and not to the microtubule polymer. γ -Tubulin was originally identified as a suppressor of a β -tubulin mutation in *Aspergillus nidulans* (Oakley and Oakley, 1989), and subsequently shown to be con-

served in all eukaryotic organisms (Stearns et al., 1991; Zheng et al., 1991; Liu et al., 1994). Although the exact mechanism of microtubule nucleation by the centrosome is not understood, several lines of evidence have implicated γ -tubulin as having an essential role in the process.

Two approaches to studying γ -tubulin have thus far yielded complementary, but largely non-overlapping, information on its function in microtubule organization. Genetic analysis in yeasts and *Aspergillus* has shown that γ -tubulin is essential for viability, is required for mitotic spindle function, and is localized to the spindle pole body (SPB),¹ the fungal equivalent of the centrosome (Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Sobel and Snyder, 1995; Marschall et al., 1996; Spang et al., 1996; Martin et al., 1997). Conditional mutations in the *Saccharomyces cerevisiae* γ -tubulin gene *TUB4* result in phenotypes that are consistent with a defect in microtubule nucleation (Marschall et al., 1996; Spang et al., 1996). In the clearest example of such a phenotype, the *tub4-34* mutant was found to be able to duplicate the SPB at the restrictive temperature, however one of the duplicated SPBs lacked

Address all correspondence to T. Stearns, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020. Tel.: (650) 725-6934. Fax: (650) 725-8309. E-mail: stearns@stanford.edu

1. *Abbreviations used in this paper:* GCP, γ -tubulin complex protein; GST, glutathione-S-transferase; SPB, spindle pole body.

microtubules, presumably because it formed at the restrictive temperature (Marschall et al., 1996). Thus, γ -tubulin function is required in yeast for the nucleation of microtubules from a new SPB but not for the continued attachment of microtubules to the SPB once they have been nucleated.

Further genetic analysis of *TUB4* led to the identification of two new proteins that associate with yeast γ -tubulin. *SPC98*, identified as a high copy suppressor of a *tub4* mutant (Geissler et al., 1996), and *SPC97*, identified as a high copy suppressor of a *spc98* mutant (Knop et al., 1997), encode essential proteins that are localized to the SPB, and physically associated with Tub4p in soluble extracts from yeast cells. The complex containing these proteins is proposed to consist of one molecule each of Spc97p and Spc98p, and at least two molecules of Tub4p (Knop and Schiebel, 1997), and to be responsible for linking microtubule ends to the SPB. Mutations in either *SPC97* or *SPC98* result in phenotypes that are similar in most respects to the *tub4* mutant phenotype (Geissler et al., 1996; Knop et al., 1997), and the localization of Spc98p is altered in a *tub4* mutant (Marschall et al., 1996), consistent with this model.

The complementary analysis of γ -tubulin in animal cells has shown that it is a component of the centrosome, but that it is also present in the cytoplasm in a protein complex much larger than that identified in yeast cells (Stearns and Kirschner, 1994). This cytoplasmic complex is required for the formation of the centrosome from centrioles (Felix et al., 1994; Stearns and Kirschner, 1994), can associate with preformed microtubules (Stearns and Kirschner, 1994; Meads and Schroer, 1995; Moudjou et al., 1996), and can nucleate microtubule polymerization (Zheng et al., 1995). Remarkably, the cytoplasmic γ -tubulin complex has the form of an open ring of the approximate diameter of a microtubule (Zheng et al., 1995). Similar ring-shaped structures containing γ -tubulin have also been detected in the pericentriolar material of centrosomes, in close proximity to microtubule ends (Moritz et al., 1995; Vogel et al., 1997). The ring-shaped γ -tubulin complex has been proposed to act directly as a template for microtubule assembly (Zheng et al., 1995), although an alternative model has been proposed (Erickson and Stoffer, 1996) in which the apparent ring is actually a coiled protofilament of γ -tubulin that acts by lateral interactions with microtubule protofilaments. The cytoplasmic γ -tubulin complex, as purified from *Xenopus* eggs, consists of at least six proteins in addition to γ -tubulin (Zheng et al., 1995). The identity and function of these other components is not known, nor is their relationship to the yeast proteins that have been identified as interacting directly with γ -tubulin.

We report here the protein composition of the γ -tubulin complex in mammalian cells, and the identification of human homologues of yeast Spc97p (GCP2) and Spc98p (GCP3) as components of this γ -tubulin complex. These proteins appear to be conserved in fungi, plants, and animals. The GCP2 and GCP3 proteins are found both at the centrosome and in the soluble γ -tubulin complex. Interestingly, the Spc97p/GCP2 and Spc98p/GCP3 proteins are related to each other, suggesting that they represent a gene family of γ -tubulin-interacting proteins. These results argue strongly that the molecular mechanism of microtubule

nucleation is conserved in the divergent organizing centers represented by the animal cell centrosome and the yeast SPB.

Materials and Methods

Human γ -Tubulin Constructs

Human γ -tubulin (cDNA provided by B. Oakley [Ohio State University, Columbus, OH]) was amplified with primers that place an NcoI site at the start codon and a Sall site directly in front of the stop codon. This version of γ -tubulin was cloned into pUC118 to create pTS551. The addition of NcoI and Sall restriction sites to γ -tubulin, which allows the fusion of epitope tags to either the NH₂ or COOH terminus of γ -tubulin, resulted in the following amino acid changes; P2A, E450V, and Q451D. These changes do not appear to effect the function of γ -tubulin since epitope-tagged versions of this protein function normally (see Fig. 1). To create COOH-terminal epitope-tagged versions of γ -tubulin, the insert from pTS551 was cloned into the vectors pTS377 and pTS335, creating γ -tubulinMyc (pTS555) and γ -tubulinHA (pTS553). pTS377 and pTS335 are derivatives of pcDNA1Neo (Invitrogen, Carlsbad, CA) that have a single Myc (pTS377) or hemagglutinin (HA) (pTS335) epitope tag sequence added to the polylinker. A XhoI site near the 3' ends of the HA and Myc sequences allowed the addition of an oligonucleotide cassette coding for a contiguous stretch of six histidine residues. This resulted in plasmids containing γ -tubulinMycHis (pTS556) and γ -tubulinHAHis (pTS554).

Yeast Methods

Media and genetic methods for *Schizosaccharomyces pombe* were as described (Moreno et al., 1991). Wild-type human γ -tubulin, γ -tubulinMycHis and *S. pombe* γ -tubulin (*tug1*⁺) were placed under the control of the alcohol dehydrogenase promoter in the *S. pombe* expression vector pART3, which is a derivative of pART1 (McLeod et al., 1987), to create pTS612, pTS613, and pTS253, respectively. These plasmids and pART3 vector were transformed into the *S. pombe* diploid strain TT304 *h+*/*h-*, *ade6-210/ade6-216*, *leu1-32/leu1-32*, *tug1::URA4/tug1*⁺, *ura4-D18/ura4-D18* (Stearns et al., 1991). To determine if the plasmids could complement the *tug1::URA4* null mutation, random spore analysis was performed. Briefly, diploid transformants were streaked onto sporulation plates and incubated until ~75% of the cells had sporulated. These cells were incubated overnight in glutulase (Dupont-NEN, Boston, MA) to kill unsporulated diploids. The spores were plated onto minimal medium lacking both leucine and uracil to select for cells containing the *tug1* disruption and the plasmid. Similar numbers of haploids were recovered from strains with the γ -tubulin, γ -tubulinMycHis, or *tug1*⁺ plasmids. The lack of a chromosomal copy of *tug1*⁺ gene in these haploids was confirmed by PCR.

Cell Culture

CHO cells, human 293 cells, and BALB/c 3T3 fibroblasts (subclone A31) and their derivatives were grown at 37°C with 5% CO₂. CHO cells were grown in α -MEM media (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS. A31 cells were grown in DME media with 10% newborn calf serum. 293 cells were grown in DME media with 10% FCS.

Permanent cell lines stably expressing either γ -tubulinMyc, γ -tubulinMycHis, γ -tubulinHAHis, or hGCP3MycHis under the control of the cytomegalovirus promoter were generated by transfecting A31 cells with pTS555, pTS556, pTS56,0 and pTS717, respectively. The A31 cells were transfected by the calcium phosphate method followed by clonal selection in the presence of 0.4 mg/ml geneticin (Gibco Laboratories). Clonal cell lines were selected for further analysis based on the expression level of the transgene as judged by immunoblot analysis and immunofluorescence using anti-HA (12CA5; Green et al., 1987) or anti-Myc (9E10; Evan et al., 1985) antibody as appropriate.

Immunofluorescence Methods

A31 cells were prepared for immunofluorescence by growing on acid-washed, poly-L-lysine treated coverslips for 2–3 d. The cells were fixed in –20°C methanol for 5 min, hydrated in PBS for 1 min, and then blocked in buffer A (3% BSA in PBS plus 0.1% Triton X-100 and 0.02% azide) for 45 min. CHO cells were similarly prepared except 0.2% gelatin was substituted for BSA in buffer A. Primary antibodies were diluted as follows: anti-Myc (mouse monoclonal 9E10) diluted 1:200; anti- γ -tubulin (affinity-

purified rabbit polyclonal XGC-1-4 [Stearns and Kirschner, 1994] diluted 1:200; anti- γ -tubulin (mouse monoclonal GTU-88; Sigma Chemical Co., St. Louis, MO) diluted 1:1000; anti- α -tubulin (rat monoclonal YL1/2; Accurate Chemical and Scientific Corp., Westbury, New York), 1:200; anti- α -tubulin (mouse monoclonal DM1 α ; Blose et al., 1984) diluted 1:1000; anti-hGCP2 (affinity-purified rabbit polyclonal) diluted 1:2; anti-hGCP3 (affinity-purified rabbit polyclonal) diluted 1:25. Primary antibody incubations were for 30 min to 1 h, followed by three washes with buffer A. Secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and were diluted 1:200 in buffer A. Secondary antibody incubations were for 30 min. to 1 h, followed by three washes with buffer A. Coverslips were stained with 1 μ g/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Molecular Probes, Eugene, OR) for 1 min, washed three times in PBS, and then mounted on slides with 90% glycerol PBS, pH 9.0, plus 1 mg/ml *para*-phenylenediamine. In experiments using anti-hGCP2 or anti-hGCP3 the BSA in buffer A was replaced with 0.2% gelatin for all incubations and washes, which resulted in lower background staining. Micrographs were taken with a Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) on Kodak TMY film.

Lysate Preparation and Sucrose Gradient Analysis

Cells were washed twice in HBS (50 mM Hepes, pH 7.4, 150 mM NaCl) at 4°C and collected by scraping. The cell pellet was either frozen in liquid nitrogen and then lysed, or used directly. The cell pellet was resuspended in three volumes of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.4, 2 mM EDTA, 1 mM DTT, 0.5% Triton X-100, aprotinin, leupeptin, pepstatin, and PMSF), incubated for 10 min on ice, and then centrifuged for 10 min at 12,000 g at 4°C to clarify. The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

2.2 ml 10–40% linear sucrose gradients were made in 50 mM Hepes, pH 7.4, 150 mM NaCl, and 2 mM EDTA with a Hoefer gradient maker (Pharmacia Biotechnology Inc., Piscataway, NJ). 100 μ l of cell lysate was loaded on the sucrose gradient and centrifuged for 2.5 h at 55,000 rpm (~100,000 g) in a TLS-55 rotor in a ultracentrifuge (TL-100; Beckman Instruments, Inc., Fullerton, CA) at 4°C. To determine the sedimentation coefficient, a parallel gradient was run with standards consisting of thyroglobulin (19S), catalase (11S), aldolase (7S), BSA (4.5S), and ovalbumin (3.6S) (Pharmacia Biotechnology Inc.).

The soluble/insoluble ratios for GCP2, GCP3, and γ -tubulin were determined by centrifuging whole cell lysate (prepared as above, but without the clarifying centrifugation) through a 10–40% sucrose gradient as described above. The amounts of GCP2, GCP3, and γ -tubulin in the 32S peak (soluble) and in the pellet (insoluble) were determined by immunoblotting, followed by densitometric quantification.

Immunoblotting

Protein samples were separated on 7.5% SDS–polyacrylamide gels, electrophoretically transferred to nitrocellulose, and then blocked for 45 min in 4% nonfat dried milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20). Primary antibody incubations were followed by incubation with HRP-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch Laboratories, Inc.). Immunoreactive proteins were visualized by chemiluminescence (Amersham International, Buckinghamshire, UK).

Metabolic Labeling of Cell Lines

Cells were grown to ~50% confluence at which time fresh newborn calf serum was added to the media (10% final volume) to ensure robust growth. 12 h before labeling the media was changed to 90% DME without L-methionine and L-cysteine, plus 10% dialyzed calf serum, 10% normal media (containing methionine and cysteine), and ~150 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine (protein labeling mix; Dupont-NEN, Boston, MA). The cells were allowed to grow in the presence of [³⁵S]methionine and [³⁵S]cysteine for ~8 h, and then fresh complete media was added to 10% final volume, and incubation continued for another 7 h. Cells were collected and lysed as described above, except that the cells were resuspended in 150 μ l of lysis buffer. The lysate was loaded on 2.1 ml 10–40% sucrose gradients and centrifuged as described above. Fractions containing the γ -tubulin complex were immunoprecipitated as described below. Immunoprecipitates were collected without boiling (see below) and analyzed on a 7.5% SDS–polyacrylamide gel. The gel was impregnated with

the autoradiography enhancer, En3hance (Dupont-NEN, Boston, MA), dried, and then visualized by fluorography.

Immunoprecipitations

Cell lysates were prepared as described above and equal amounts of protein lysate from each cell line were added to two eppendorf tubes containing protein A–Sepharose (Sigma Chemical Co.), the appropriate antibody (anti-HA, anti-Myc, or both) and in one tube of each pair an ~10-fold molar excess of antigenic peptide (i.e., the 12CA5 peptide, the 9E10 peptide, or both). The immunoprecipitations were performed at 4°C for 1.5 h with mixing. The immunoprecipitates were washed once in lysis buffer, twice in 250 mM NaCl (250 mM NaCl had no effect on the size of the γ -tubulin complex), 50 mM Hepes, pH 7.4, and once in HBS. 175 μ l of 1 \times sample buffer (62.4 mM Tris, pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 2.3% SDS, 0.01% bromophenol blue) was added to each tube and incubated for 5 min at room temperature, and then split into three tubes. Samples to be probed with anti-hGCP2 or anti-hGCP3 were boiled for 5 min, whereas samples to be probed with anti- γ -tubulin were incubated for another 5 min at room temperature. Incubation at room temperature was found to dissociate the antibodies from protein A but not dissociate the two heavy chains of the antibody, resulting in the heavy chains migrating at a molecular weight of ~100–110 kD in SDS-PAGE (the heavy chains from the anti-Myc antibody migrate slightly more rapidly than the heavy chains from the anti-HA antibody). Incubation at 100°C caused the antibody heavy chains to dissociate and migrate at ~50–55 kD in SDS-PAGE analysis. Manipulation of the heavy chain migration ensured that the large amounts of heavy chain on the gel did not affect the mobility of the protein(s) being examined.

Cloning of hGCP2 and hGCP3

A human EST (these sequence data are available from Genbank/EMBL/DDBJ under accession No. T55505) was identified that had a predicted protein product with low, but significant, homology to SPC98. PCR primers hGCP3.4 and hGCP3.5 based on this EST were used to amplify an ~210-bp fragment from a human B-cell library (Durfee et al., 1993).

hGCP3.4 GCGGATCCATTCTAGAAACCGCTGTACAGACCC
hGCP3.5 CCATCGATGAATCCCTCCAGAGGAAGTTAAATAC

This PCR product was cloned into pGEM 3 Zi(+) (Promega, Madison, WI) to create pTS761. The above primers were used with pTS761 in a primer extension reaction in the presence of [α -³²P]dCTP to make a radiolabeled probe (Sambrook et al., 1989). This probe was used to screen 5 \times 10⁵ plaques from a lambda ZAP2 HeLa cell cDNA library (Stratagene, La Jolla, CA) by hybridization. The longest insert of 25 positive phages was sequenced in its entirety on both strands except for the extreme 3' untranslated region. The sequence that was obtained lacked a start codon. One cDNA with a longer 5' end was identified among the original positive clones by PCR, sequenced, and then shown to have a putative start codon plus ~100 bp of 5' untranslated sequence. Although there is no stop codon upstream of this putative start codon, the *Xenopus* homologue of this protein uses the same start codon (Martin et al., 1998), thus it is likely to be correct.

A version of hGCP3 containing a SalI site in place of the stop codon was generated by replacing the 3' end of hGCP3, from the KpnI site to the stop codon, with an oligonucleotide cassette containing a SalI site in place of the stop codon. This construct was cloned into pcDNA1Neo along with the MycHis tag from pTS556. This resulted in the fusion of the MycHis epitope tag to the COOH terminus of hGCP3 (hGCP3MycHis) in pTS718.

hGCP2 was cloned by an approach similar to that described above for hGCP3. Six human ESTs were found that had a predicted protein product with low but significant, homology to a region of Spc97p. PCR primers hGCP2.1 and hGCP2.2 based on these ESTs were used to amplify a 366-bp fragment from a human hepatoma cDNA library (Schild et al., 1990).

hGCP2.1 GGAAGATCTTCGCTGCACAGGCAGGGCCCTC
hGCP2.2 CGGAATCCATCGATGCTAGATGGTGTACCGCTGGT

The resulting PCR product was cloned into pGEX-2T (Pharmacia Biotechnology Inc.) to create pTS762. The above primers were used to make a radiolabeled version of this fragment. This probe was used to screen 5 \times 10⁵ lambda from a lambda ZAP2 HeLa cell cDNA library. Of the 18 positive phages isolated from this screen, seven were converted to plasmids in the bacterial strain Able K (Stratagene), which maintains Bluescript-

based plasmids at a low copy number. We were unable to excise plasmids using SOLR bacteria, which maintains Bluescript-based plasmids at a high copy number, suggesting that the cDNA for hGCP2 is somewhat toxic in bacteria. Only one of these plasmids was stably maintained in bacteria. hGCP2 was sequenced from this plasmid on one strand with a Thermo Sequenase dye terminator cycle sequencing kit (Amersham Life Science). The sequence obtained experimentally for hGCP2 was consistent with that obtained independently by combining many overlapping human ESTs.

Antibodies

A 15-amino acid peptide (CEVSPGDTGWDVFSLD) was synthesized (Research Genetics Inc., Huntsville, AL) to correspond to amino acids 627–641 of hGCP3. A cysteine residue was added to the NH₂ terminus to allow cross-linking of this peptide to a carrier protein and the COOH terminus was amidated. This peptide was coupled to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) with Sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Pierce Chemical Co., Rockford, IL), as per manufacturer's instructions, and injected into two rabbits for antibody production (BAbCO, Richmond, CA). The resulting antibodies were affinity-purified against a glutathione-S-transferase (GST) hGCP3 fusion protein made by cloning hGCP3 into pGEX-2T to create pTS675. Expression of the GST-hGCP3 protein was induced by addition of IPTG to 0.5 mM. The fusion protein is insoluble and was isolated as inclusion bodies (Harlow and Lane, 1988). Inclusion bodies were resuspended in sample buffer and analyzed on a 10% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose and used to affinity purify the anti-hGCP3 antibodies (Harlow and Lane, 1988).

A region of hGCP2 corresponding to amino acids 312–434 was cloned into pGEX-2T to create pTS762. The resulting GST-hGCP2 fusion protein was isolated as inclusion bodies and analyzed on a 10% SDS-polyacrylamide gel. Polyacrylamide gel slices containing the fusion protein were used to immunize two rabbits for antibody production (BAbCO). The same fragment of hGCP2 was cloned into pATH11 (Dieckmann and Tzagoloff, 1985) to create pTS763. The resulting TrpE-hGCP2 fusion protein was used to affinity-purify the anti-hGCP2 antibodies by two different methods. Insoluble TrpE-hGCP2 fusion protein was solubilized and either analyzed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose, or coupled to Affi-gel 10 matrix in the presence of 0.05% SDS. Antibodies purified by binding to and elution from nitrocellulose bearing the TrpE-hGCP2 fusion protein (Harlow and Lane, 1988) were used for Figs. 5, 7, and 9. Antibodies purified by column chromatography (Harlow and Lane, 1988) on the coupled Affi-gel matrix were used in Figs. 6 and 10.

Baculovirus Construction and Expression of Proteins in Sf9 Cells

hGCP2 and hGCP3 were cloned into the baculovirus transfer vector pVL1392 (O'Reilly et al., 1992). The resulting plasmids were individually cotransfected with Baculogold (PharMingen, San Diego, CA) into Sf9 cells. Recombinant baculoviruses were isolated and plaque-purified as previously described (Morgan et al., 1991). hGCP2 and hGCP3 baculoviruses were used to infect Sf9 cells as previously described (Morgan et al., 1991). The infected cells were collected after 2 d and lysed in the lysis buffer described above. The insoluble pellet was resuspended in sample buffer.

Microtubule-binding Assay

Bovine brain tubulin (Cytoskeleton, Inc., Denver, CO) was diluted to a final concentration of 1 mg/ml in microtubule stabilizing buffer BRB80 (80 mM Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) plus 1 mM GTP, 10% glycerol, and 50 µg/ml rhodamine-conjugated bovine brain tubulin, prepared as described (Hyman et al., 1991). The tubulin was incubated with increasing amounts of taxol (Calbiochem-Novabiochem Corp., San Diego, CA), 0.1 µM for 2 min, 1 µM for 2 min, 10 µM for 30 min at 37°C, to induce microtubule polymerization. After incubation, the samples were examined for the presence of microtubules by fluorescence microscopy. The average length of the microtubules in the taxol-treated sample was ~5 µm. Cytoplasmic cell lysate was made from A31 cells as described above except that the lysis buffer contained EGTA instead of EDTA, 1 mM GTP, and 100 µg/ml cytochalasin B. Taxol-stabilized microtubules were added to the lysate to obtain a final concentration range of 0–500 pM microtubules. All of the samples were incubated at 30°C for 30 min, and then each sample was

loaded on top of a 1-ml cushion of 40% glycerol in BRB80 and 1 mM GTP (plus taxol in all samples with assembled microtubules). The samples were centrifuged for 10 min at 30°C in a TLS-55 rotor (Beckman Instruments, Inc., Fullerton, CA) at 50,000 rpm. The pellets were resuspended in sample buffer and analyzed on 7.5% SDS-polyacrylamide gels. Samples were analyzed by both Coomassie blue staining and immunoblotting with anti-γ-tubulin, anti-hGCP2, and anti-hGCP3 antibodies.

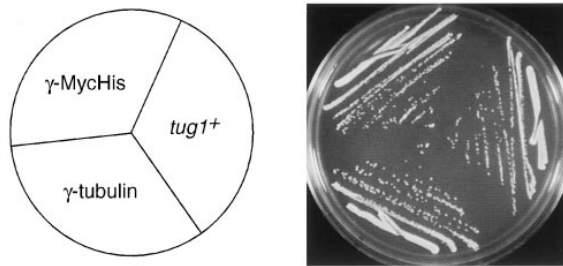
Results

The Composition of the γ-Tubulin Complex Is Similar in Mammals and Frogs

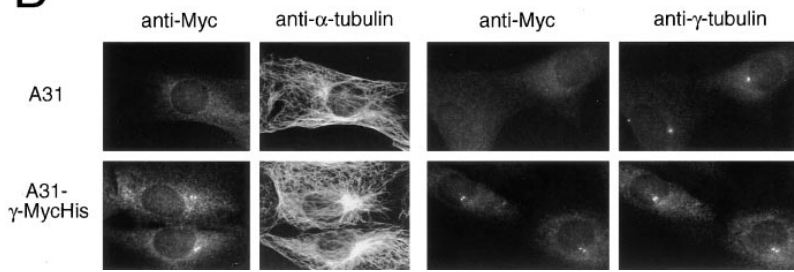
To examine the composition of the mammalian cytoplasmic γ-tubulin complex, mouse BALB/c 3T3 cell lines were generated that stably express various epitope-tagged versions of human γ-tubulin. γ-Tubulin was epitope-tagged on its COOH terminus by fusing either a peptide from the myc protein (γ-tubulinMyc), the myc peptide plus a contiguous stretch of six histidine residues (γ-tubulinMycHis), or a peptide from the hemagglutinin (HA) protein plus a contiguous stretch of six histidine residues (γ-tubulinHAHis). The function of these tagged γ-tubulin derivatives was tested in several ways. First, epitope-tagged γ-tubulin was tested for its ability to complement a γ-tubulin null mutation in the fission yeast, *Schizosaccharomyces pombe*. The *S. pombe* γ-tubulin (*tug1*⁺) gene is essential for viability in *S. pombe* (Stearns et al., 1991), but this requirement can be satisfied by the expression of either *tug1*⁺ or human γ-tubulin (Horio and Oakley, 1994). Wild-type human γ-tubulin, γ-tubulinMycHis, and *tug1*⁺ each fully complemented a *tug1*⁺ null mutation (Fig. 1 A). Second, the ability of epitope-tagged γ-tubulin to localize to the centrosome and be incorporated into the cytoplasmic γ-tubulin complex in animal cells was examined. γ-TubulinMycHis localized to the centrosome as evidenced both by colocalization with endogenous γ-tubulin, and localization to the focus of microtubules in a mouse BALB/c 3T3 (subclone A31) cell line stably expressing γ-tubulinMycHis (Fig. 1 B). Finally, γ-tubulinMycHis and γ-tubulinHAHis (not shown) incorporated into the cytoplasmic γ-tubulin complex, as evidenced by their cosedimentation with endogenous γ-tubulin in sucrose gradients (Fig. 1 C). The epitope-tagged γ-tubulin derivatives could be distinguished from endogenous γ-tubulin by their slower mobilization on SDS-PAGE gels.

The cytoplasmic γ-tubulin complex in A31 cells sedimented with the same velocity as that in *Xenopus* egg extracts, with a sedimentation coefficient of ~32S. The difference in S value from our previously published results (Stearns and Kirschner, 1994) is due to a more accurate determination here, and is similar to that reported for a different mammalian cell line (Meads and Schroer, 1995). In the A31 γ-tubulinMycHis cell line shown in Fig. 1 C, the level of γ-tubulinMycHis is lower than the level of endogenous γ-tubulin. In many such cell lines examined, none were found that expressed the transfected gene at higher levels than the endogenous gene, suggesting that overexpression of γ-tubulin is detrimental. This contrasts with the case in yeast, in which γ-tubulin can be overexpressed 300-fold without phenotypic effect (Marschall et al., 1996), but is consistent with transient transfection results in animal cells, showing that an ~100-fold overexpression of

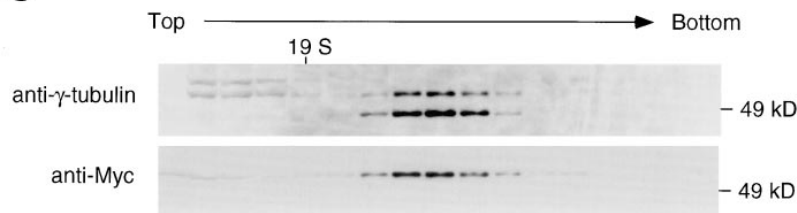
A



B



C



γ -tubulin caused a disruption of the microtubule cytoskeleton (Shu and Joshi, 1995).

The composition of the γ -tubulin complex was determined by examining the proteins that co-immunoprecipitate with epitope-tagged γ -tubulin. To visualize these proteins, cell lines expressing epitope-tagged γ -tubulin were metabolically labeled with [35 S]methionine and [35 S]cysteine. Soluble extracts from these cells were then run on a sucrose gradient, and epitope-tagged γ -tubulin was immunoprecipitated from the fractions containing the peak of γ -tubulin protein. To be judged a genuine member of the γ -tubulin complex, the immunoprecipitation of candidate proteins must (a) be blocked by the antigenic peptide corresponding to the epitope tag, (b) be dependent on the expression of the epitope-tagged γ -tubulin (i.e., must not occur in the parental cell line), and (c) occur with both the Myc- and HA-tagged γ -tubulin derivatives. Using these criteria, seven proteins of 48, 71, 76, 100, 101 (doublet with 100), 128, and 211 kD specifically coimmunoprecipitated with epitope-tagged γ -tubulin (Fig. 2). The mobility of the coimmunoprecipitating bands of \sim 100 and 101 kD is altered when the complex is immunoprecipitated by the anti-Myc antibody because the heavy chains of the anti-Myc antibody migrate at \sim 100 kD in this experiment (see Materials and Methods). The position of the epitope-tagged γ -tubulins are indicated with an asterisk; the mobility of this band is altered depending on the size of the

Figure 1. Behavior of epitope-tagged γ -tubulin. (A) Epitope-tagged γ -tubulin complements a null mutation in *S. pombe*. The growth of haploid *tug1::URA4* strains bearing plasmids expressing the indicated γ -tubulin genes was assayed on rich medium. (B) γ -TubulinMycHis is localized to the centrosome. Immunofluorescence was performed on the parental A31 cell line and a cell line expressing γ -tubulinMycHis (A31- γ -MycHis). The cells were co-stained with anti-Myc and either anti- α -tubulin or anti- γ -tubulin. (C) γ -tubulinMycHis cosediments with the γ -tubulin complex in sucrose gradients. Cytoplasmic extract from the A31- γ -tubulinMycHis cell line was sedimented through a sucrose gradient and gradient fractions were immunoblotted with anti- γ -tubulin and anti-Myc antibodies. The position at which thyroglobulin (19S) sedimented in a gradient run in parallel is indicated, as is the migration of the 49-kD prestained molecular weight marker. Note that γ -tubulinMycHis migrates more slowly than endogenous γ -tubulin.

epitope tag (Fig. 2). The molecular weight of the 48-kD coimmunoprecipitating band suggested that it might represent wild-type γ -tubulin. To test this hypothesis, immunoprecipitates from the epitope-tagged γ -tubulin cell lines were immunoblotted with an anti- γ -tubulin antibody. The anti- γ -tubulin antibody recognized both the epitope-tagged γ -tubulin and the 48-kD band, thus identifying it as the endogenous γ -tubulin (Fig. 3).

The molecular weights of the mammalian γ -tubulin complex components as defined above are similar to those reported previously for the *Xenopus* γ -tubulin complex (Zheng et al., 1995), and for γ -tubulin complexes identified in sheep brain extracts (Detraves et al., 1997) (Table I). Whereas both of these other groups identified α - and β -tubulin as components of the γ -tubulin complex, we observed no bands corresponding to these proteins in the immunoprecipitates from metabolically labeled cells. Immunoblotting of immunoprecipitates with anti- α -tubulin antibodies similarly failed to reveal any α -tubulin in the γ -tubulin complex (data not shown), even under buffer conditions similar to those used in the experiments that did detect α - and β -tubulin.

Cloning of the Human Homologues of Yeast Spindle Pole Components Spc98p and Spc97p

During the course of the above experiments, genetic ex-

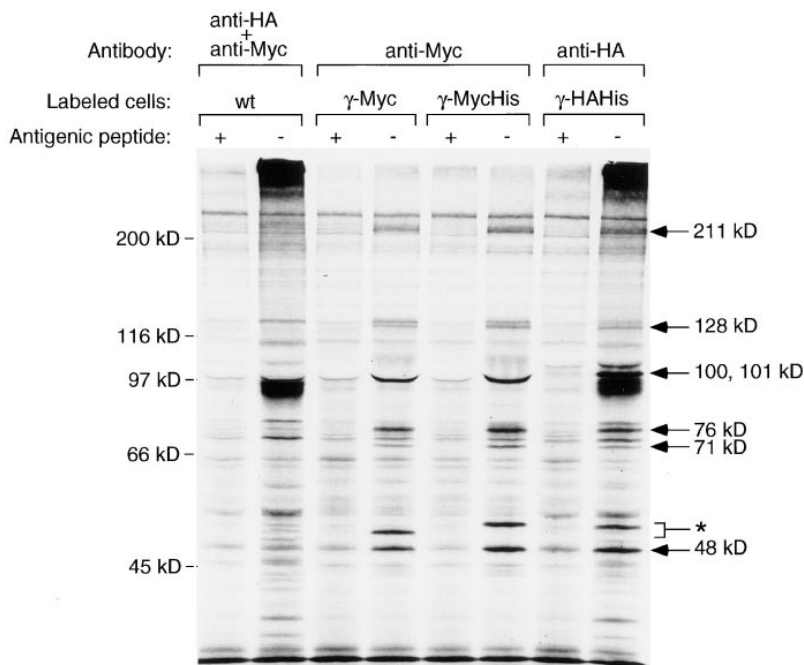


Figure 2. The composition of the mouse γ -tubulin complex. A31 (*wt*), and A31-derived cell lines expressing γ -tubulinMyc, γ -tubulinMycHis, and γ -tubulinHAHis were metabolically labeled with [35 S]methionine and [35 S]cysteine. Cytoplasmic extracts from these cells were sedimented through sucrose gradients and the epitope-tagged γ -tubulins were immunoprecipitated from fractions containing the γ -tubulin complex with the indicated antibody in the presence (+) or absence (-) of competing antigenic peptide. The resulting immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gel and visualized by fluorography. Molecular weight markers are indicated on the left. The arrows and numbers on the right indicate the position and calculated molecular weight of proteins that specifically coimmunoprecipitate with γ -tubulin. The position of the epitope-tagged γ -tubulins is indicated by the asterisk. The migration of the 100-, 101-kD doublet is altered when the anti-Myc antibody is used because the heavy chains of this antibody migrate at \sim 100 kD (see Materials and Methods).

periments in *Saccharomyces cerevisiae* identified a protein, Spc98p, that interacted with yeast γ -tubulin (Geissler et al., 1996). To examine the possibility that a homologue of this yeast γ -tubulin-associated protein might exist and be a component of the animal γ -tubulin complex, we searched the expressed sequence tag (EST) database for Spc98p-related proteins. Human ESTs showing limited but significant homology with Spc98p were identified, and used to isolate a full-length cDNA from a HeLa cell cDNA library. When this human sequence was used to search the yeast genome database, it was found that there were two yeast proteins with significant homology. One was Spc98p, the other was an unknown protein that was subsequently identified as the γ -tubulin interacting protein Spc97p (Knop et al., 1997). This result led us to search for a human homologue of Spc97p. As with Spc98p, human ESTs showing limited but significant homology with Spc97p were identified, and used to isolate a full-length cDNA from a HeLa cell cDNA library. There were multiple hu-

man EST sequences corresponding to each of the genes, and these ESTs came from diverse tissues and cell types, suggesting that both genes are ubiquitously expressed.

The open reading frames of the human homologues of Spc97 and Spc98 predicted proteins of 103 and 104 kD, respectively. In light of evidence presented below that these proteins are components of the γ -tubulin complex, we propose a naming convention for these and other members of the complex. γ -Tubulin itself would be γ -tubulin complex protein (GCP) 1, the homologue of Spc97p would be GCP2, and the homologue of Spc98p would be GCP3. These names eliminate the confusion caused by names based on the molecular weight, as these will differ among organisms. When referring specifically to the human genes or proteins, we will refer to them as hGCP2 and hGCP3. (The sequence data for hGCP2 and hGCP3 are available from GenBank/EMBL/DBJ under accession numbers AF042379 and AF042378, respectively.) A portion of the gene for hGCP3 was previously identified as an expressed

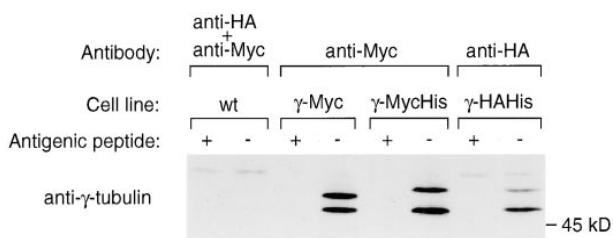


Figure 3. There are multiple γ -tubulin molecules in the γ -tubulin complex. Epitope-tagged γ -tubulin was immunoprecipitated from cytoplasmic extracts of the indicated cell lines in the presence and absence of antigenic peptide. These immunoprecipitates were analyzed on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and then immunoblotted with anti- γ -tubulin antibodies. The migration of a 45-kD molecular weight marker is indicated.

Table I. The Composition of γ -Tubulin Complexes from Various Organisms

Mouse*	Sheep [†]	Xenopus [‡]
	<i>Mol wt (kD)</i>	
—	250	—
211	195	195
128	130	133
100, 101 (doublet)	105	109 (doublet)
76	75	75 (3–4 bands)
71	—	—
—	56/55 (α - β -tubulin)	56/55 (α - β -tubulin)
48 (γ -tubulin)	50 (γ -tubulin)	50 (γ -tubulin)

*This study.

[†]Detraives et al., 1997.

[‡]Zheng et al., 1995.

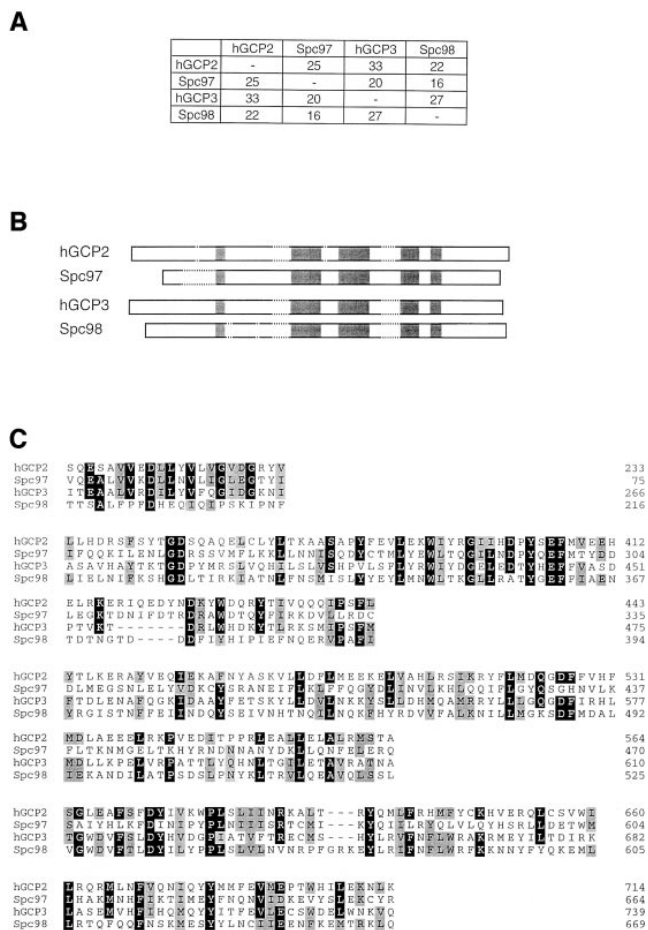


Figure 4. hGCP3, Spc98p, hGCP2, and Spc97p are related proteins. (A) Pair-wise comparison of hGCP2, Spc97p, hGCP3, and Spc98p. The numbers represent the percent similarity using the following groupings of amino acids: N and Q; R and K; S and T; D and E; Y, F, and W; I, L, M, and V; H; P; C; G; and A. (B) A schematic diagram of the hGCP2, Spc97p, hGCP3, and Spc98p proteins. The boxes representing the proteins are drawn to scale, with the dashed segments representing gaps of 10 amino acids or more that were introduced to align the proteins. The shaded regions represent regions that show significant homology. (C) The amino acid sequences of the shaded regions of homology indicated in (B). Amino acid positions that are either identical (*black shading*) or similar (*gray*) in at least three of the proteins are indicated. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF042379 (*hGCP2*) and AF042378 (*hGCP3*).

gene in human liver, and was mapped to chromosome 13, q34 (GenBank locus HUMCH13C2B).

Sequence comparisons revealed that Spc97p and hGCP2 are 25% similar to each other, and that Spc98p and hGCP3 are 27% similar to each other (Fig. 4 A). Although no homology was noted previously between the yeast Spc97p and Spc98p proteins, comparison with hGCP2 and hGCP3 revealed that all four of the proteins are related to each other. The homology is most striking in the five regions that are displayed in Fig. 4, B and C. hGCP2 and hGCP3 are more similar to each other than to either of the yeast proteins. Spc97p and Spc98p have the least similarity, but

are clearly related, particularly in the second homology region in Fig. 4, B and C.

In addition to the human ESTs in the database, we also noted the presence of ESTs from other organisms for both GCP2 and GCP3, indicating that these proteins are conserved. For GCP2 there are homologous sequences from mouse, *Drosophila*, and a moss, and for GCP3 there are homologous sequences from mouse, zebrafish, and rice. The vertebrate gene segments are all highly homologous, displaying ~90% protein sequence identity (not shown).

GCP2 and GCP3 Are Centrosomal Proteins

Antibodies were generated against a bacterially expressed portion of the hGCP2 protein, and against a peptide from hGCP3. These antibodies reacted with the human proteins, and cross-reacted with the corresponding proteins in mouse and *Xenopus* cells (not shown). Specificity of the antibodies was demonstrated by testing both affinity-purified antibodies against full-length hGCP2 and hGCP3 proteins on Western blots. Antibodies generated against hGCP2 did not cross-react with hGCP3, and antibodies generated against hGCP3 did not cross-react with hGCP2 (not shown). These affinity-purified antibodies were used in immunofluorescence experiments to determine the localization of the proteins in animal cells (Fig. 5). Both proteins localized to the centrosome, as evidenced by localization to the focus of microtubules and colocalization with γ -tubulin. The centrosomal staining in both cases was eliminated by competing antigen (not shown). Complete depolymerization of the microtubule cytoskeleton with the microtubule-depolymerizing drug nocodazole did not affect the centrosomal localization of either GCP2 or GCP3 (not shown). This indicates that both GCP2 and GCP3, like γ -tubulin (Stearns et al., 1991), are intrinsic centrosome components that do not require microtubules to maintain their localization.

GCP2 and GCP3 Are Components of the γ -Tubulin Complex

Previous experiments have shown that more than half of the total γ -tubulin in animal cells is soluble, in the form of the cytoplasmic γ -tubulin complex (Felix et al., 1994; Stearns and Kirschner, 1994; Meads and Schroer, 1995; Moudjou et al., 1996). Lysates of A31 cells were separated into soluble and insoluble fractions and tested for the presence of γ -tubulin, GCP2, and GCP3. All three proteins had a similar soluble/insoluble distribution, with >70% of the total protein being soluble. The remainder was insoluble, presumably associated with the centrosome. To determine whether GCP2 and GCP3 are components of the γ -tubulin complex, we first examined their behavior on sucrose gradients. Cytoplasmic extracts of human 293 cells were fractionated on 10–40% sucrose gradients and probed with antibodies against γ -tubulin, GCP2, and GCP3. All three proteins sedimented with the same velocity in these gradients (Fig. 6), well separated from the majority of soluble proteins. As indicated above, we estimate the sedimentation coefficient of this complex to be 32S. We next checked for a physical interaction between γ -tubulin and GCP2 and GCP3 by immunoprecipitating epitope-tagged versions of γ -tubulin from the cell lines described above

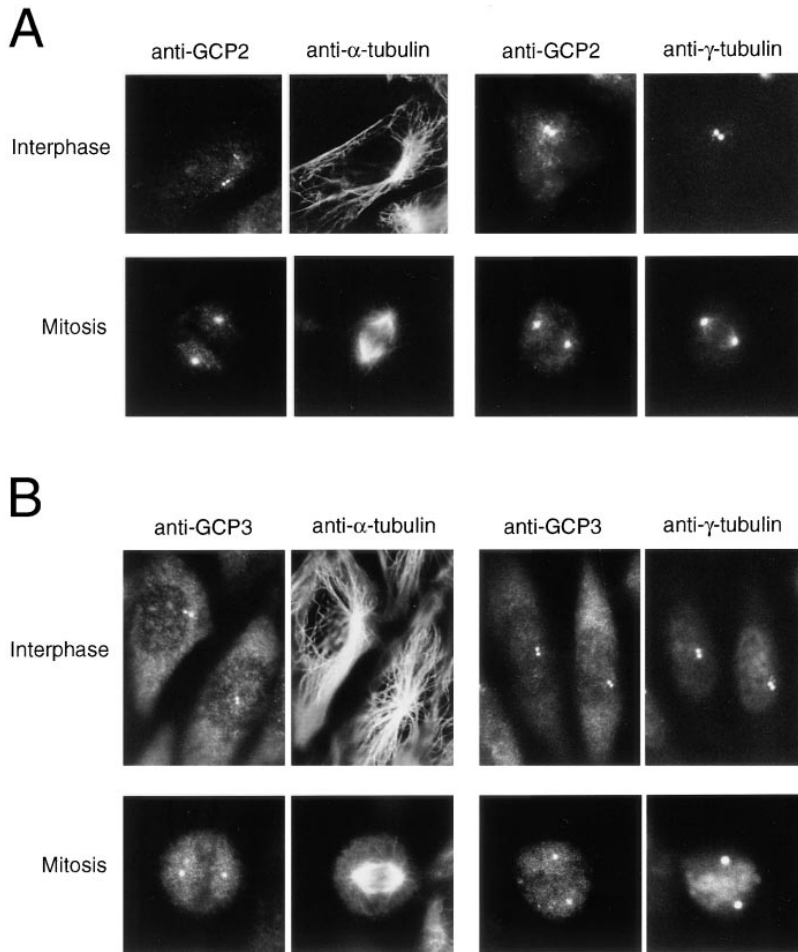


Figure 5. GCP2 and GCP3 are localized to the centrosome. (A) CHO cells were fixed and co-stained with anti-hGCP2 and either anti- α -tubulin or anti- γ -tubulin antibodies as indicated. (B) CHO cells were fixed and co-stained with anti-hGCP3 and either anti- α -tubulin or anti- γ -tubulin as indicated. Examples of cells in interphase and mitosis are shown.

and assaying the immunoprecipitates for the presence of γ -tubulin, GCP2, and GCP3 (Fig. 7). As shown above in Fig. 3, immunoprecipitation of epitope-tagged γ -tubulin resulted in precipitation of both the tagged and endogenous γ -tubulin. GCP2 and GCP3 were both immunoprecipitated along with γ -tubulin. The sedimentation and immunoprecipitation results demonstrate that GCP2 and GCP3 are components of the γ -tubulin complex.

Among the proteins identified in Fig. 2 as components of the γ -tubulin complex, GCP2 and GCP3 are closest in molecular weight to the 101-kD band, which runs as a very tight doublet. To determine whether the GCP2 and GCP3 proteins correspond to the 101-kD doublet, the GCP2 and GCP3 genes were cloned into baculovirus vectors and expressed in insect cells. In both cases a portion of the expressed protein was insoluble and relatively pure; this protein was used to assess mobility on Coomassie-stained gels. GCP2 and GCP3 were found to run as a tight doublet of ~ 100 kD (Fig. 8). To compare the mobility of these proteins with those of the proteins of the γ -tubulin complex, samples of GCP2 and GCP3 were run adjacent to the immunoprecipitates from labeled cells shown in Fig. 2. The Coomassie-stained GCP2 and GCP3 protein doublet comigrated with the 101-kD γ -tubulin complex components (not shown). Thus, GCP2 and GCP3 appear to be the proteins that make up the doublet at 101 kD in the γ -tubulin complex.

The γ -Tubulin Complex Contains Multiple Molecules of GCP3

To begin to investigate the stoichiometry of components in the γ -tubulin complex, we created stable cell lines expressing an epitope-tagged version of hGCP3. In this cell line, A31-hGCP3MycHis, the epitope-tagged protein localizes to the centrosome (Fig. 9 A), and cosediments with the endogenous GCP3 on sucrose gradients (Fig. 9 B). These results confirm that the cloned GCP3 gene corresponds to the centrosomal protein identified by the anti-GCP3 antibody. The level of expression of the tagged version of GCP3 is lower than that of the endogenous protein (Fig. 9 B). It was not possible to recover cell lines expressing higher levels, suggesting that overexpression of hGCP3 is toxic, similar to the situation for Spc98p in yeast (Geissler et al., 1996). Immunoprecipitation of hGCP3MycHis with anti-Myc antibody resulted in precipitation of both the endogenous and tagged proteins, indicating that the γ -tubulin complex contains more than one GCP3 molecule (Fig. 9 C). In addition, both γ -tubulin and GCP2 coimmunoprecipitate with hGCP3MycHis, confirming that these proteins are all part of the same complex (Fig. 9 C).

GCP2 and GCP3 Bind to Microtubules as Part of the γ -Tubulin Complex

Previous experiments have shown that the γ -tubulin com-

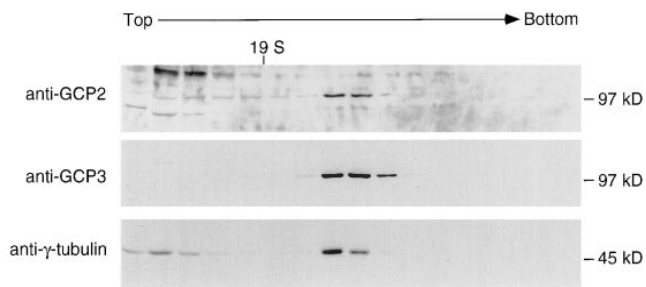


Figure 6. GCP2 and GCP3 cosediment with γ -tubulin in sucrose gradients. The sedimentation velocity of cytoplasmic γ -tubulin, GCP2, and GCP3 from human 293 cells was examined by sucrose gradient velocity sedimentation. Gradient fractions were immunoblotted with either anti- γ -tubulin, anti-hGCP2, or anti-hGCP3 antibodies. The position of thyroglobulin (19S) in a gradient run in parallel is indicated, as is the migration of the 45- and 97-kD molecular weight markers.

plex associates with microtubules in an end-dependent manner (Li and Joshi, 1995; Zheng et al., 1995), but have not determined the composition of the complex after binding. To determine whether GCP2 and GCP3 remain associated with γ -tubulin during microtubule binding, a microtubule sedimentation assay was performed. Increasing amounts of taxol-stabilized microtubules were added to cytoplasmic extracts of A31 cells, incubated, and then centrifuged to precipitate microtubules and associated proteins. The pellets were assayed for the presence of γ -tubulin, GCP2, and GCP3. The range of microtubule concentrations chosen was such that the highest number of microtubule ends used would approximately equal the number of γ -tubulin complexes in these lysates. Under these conditions, γ -tubulin sediments in a manner dependent on microtubule concentration (Stearns and Kirschner, 1994). We found that γ -tubulin, GCP2, and GCP3 sedimented with microtubules, and that all three proteins exhibited

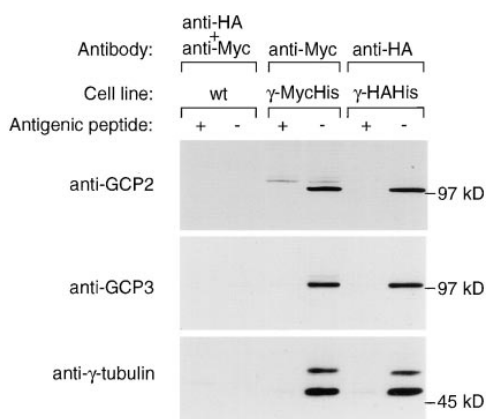


Figure 7. GCP2 and GCP3 are components of the cytoplasmic γ -tubulin complex. Epitope-tagged γ -tubulin was immunoprecipitated from cytoplasmic extracts of the indicated cell lines, with the indicated antibody in the presence or absence of competing antigenic peptide. Immunoprecipitates were immunoblotted with either anti- γ -tubulin, anti-hGCP2, or anti-hGCP3 antibodies. The positions of the 45- and 97-kD molecular weight markers are indicated.

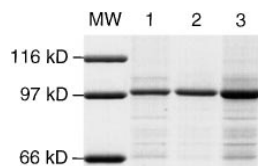


Figure 8. hGCP2 and hGCP3 migrate with the same electrophoretic mobility as the 100-, 101-kD doublet of the γ -tubulin complex. hGCP2 and hGCP3 were produced in insect cells using the baculovirus system. hGCP2 and hGCP3 were purified as insoluble proteins and analyzed on a 7.5% SDS-polyacrylamide gel, as follows: MW, molecular weight markers; 1, hGCP2; 2, hGCP3; and 3, hGCP2 plus hGCP3. The gel was stained with Coomassie blue to visualize the proteins. The molecular weights of the markers are indicated.

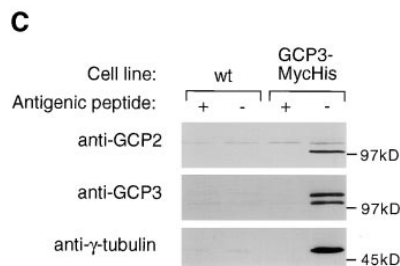
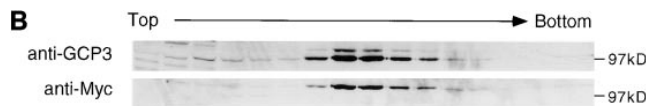
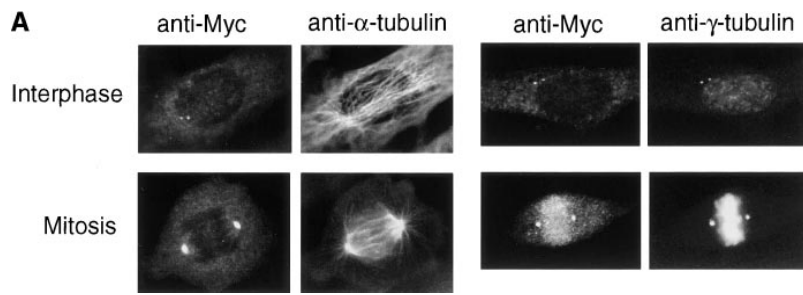
similar dependence on microtubule concentration (Fig. 10). The simplest interpretation of these results is that GCP2 and GCP3 associate with microtubules as part of the γ -tubulin complex.

Discussion

Microtubule organizing centers from different species differ dramatically in morphology, yet all carry out the basic functions of microtubule nucleation and organization. One of the few known common components of organizing centers is γ -tubulin, thought to play a critical role in microtubule nucleation. We have shown that γ -tubulin in animal cells is associated with two proteins, GCP2 and GCP3, that are homologues of the yeast spindle pole body components Spc97p and Spc98p. Like γ -tubulin, GCP2 and GCP3 are present at both the centrosome and in a cytoplasmic complex. The conservation of both γ -tubulin and these associated proteins suggests that the mechanism of microtubule nucleation will be similarly conserved.

The γ -Tubulin Complex in Yeast and Animals: Differences and Similarities

The γ -tubulin complex has been most well characterized in yeast, *Xenopus* eggs, and mammalian cells and tissues. In yeast, the complex is relatively small, running at 6S on sucrose gradients and consisting exclusively of γ -tubulin (Tub4p), Spc97p, and Spc98p. Although Spc97p and Spc98p are related, as shown here, they are both essential genes, and seem to be part of the same complex in that immunoprecipitation of one precipitates the other (Geissler et al., 1996; Knop et al., 1997). As there is no evidence for higher order forms of the γ -tubulin complex in yeast, it seems likely that the complex of γ -tubulin with Spc97p/GCP2 and Spc98p/GCP3 represents the minimal unit of the γ -tubulin complex. In animal cells the complex is much larger, \sim 32S, and we have shown that for both γ -tubulin and GCP3, tagged and untagged molecules exist in the same γ -tubulin complex. This is consistent with the estimated stoichiometry of 10–13 γ -tubulin molecules per γ -tubulin complex in frogs reported by Zheng et al. (1995). Thus, the simplest model for the large animal cell complex is that it consists of multiple γ -tubulin/GCP2/GCP3 subunits. These subunits would be joined together such that they form a ring or coiled filament. There are at least four proteins other than γ -tubulin, GCP2, and GCP3 in the γ -tubulin complex, and we propose that these proteins play a role in joining the γ -tubulin/GCP2/GCP3 subunits



together, and possibly in attaching the entire γ -tubulin complex to the centrosome.

Although the difference in size and complexity of the γ -tubulin complex in yeast and animal cells is striking, it may be only superficial. For example, it is possible that the yeast γ -tubulin/Spc97p/Spc98p subunits are monomeric in the cytoplasm, but that they assemble into ring-shaped structures upon binding to the SPB. The possibility of dynamic assembly of γ -tubulin into larger structures is suggested by previous experiments in which the *Xenopus* γ -tubulin complex was reported to be dissociated by high salt, but could reform upon returning the salt to physiological levels (Stearns and Kirschner, 1994). Meads and Schroer (1995) reported that γ -tubulin in cultured epithe-

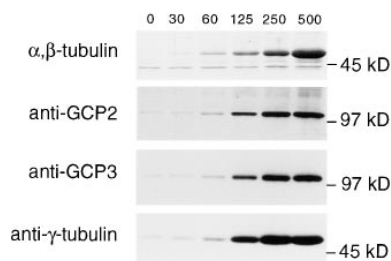


Figure 10. GCP2 and GCP3 associate with microtubules. Equal amounts of cytoplasmic lysate from A31 cells were incubated with varying concentrations of taxol-stabilized microtubules (0–500 pM microtubules). The highest concentration of microtubules was approximately equal to the predicted concentration of γ -tubulin complexes in these lysates. The samples were centrifuged to sediment microtubules and associated proteins. The pellets were then stained with Coomassie blue to visualize α - and β -tubulin, and immunoblotted with anti-hGCP2, anti-hGCP3, and anti- γ -tubulin antibodies. The positions of the 97- and 45-kD molecular weight markers are indicated.

Figure 9. Epitope-tagged hGCP3 localizes to the centrosome and incorporates into the cytoplasmic γ -tubulin complex. (A) A31 cells stably expressing hGCP3MycHis were fixed and co-stained with anti-Myc and either anti- α -tubulin or anti- γ -tubulin, as indicated. Examples of both mitotic and interphase cells are shown. (B) Cell lysates made from A31-hGCP3MycHis were run on a sucrose gradient and fractions from this gradient were immunoblotted with anti-hGCP3 and anti-Myc antibodies. Note that epitope-tagged hGCP3 migrates more slowly than endogenous GCP3. (C) Cell lysates made from A31 or A31-hGCP3MycHis cells were subjected to immunoprecipitation with anti-Myc antibodies in the presence or absence of competing antigenic peptide. The resulting immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gels and immunoblotted with anti-hGCP2, anti-hGCP3, and anti- γ -tubulin as indicated. The positions of the 97- and 45-kD molecular weight markers are indicated.

lial cells was found in two cytoplasmic forms, one 10S, the other 29S, the larger presumably representing the full-size complex. Similarly, the size of the γ -tubulin complex in *Aspergillus* is reported to range from 8S to 20S (Akashi et al., 1997), and to be sensitive to salt concentration.

An alternative possibility for the differences between yeast and animal cells is that the animal γ -tubulin complex has evolved to perform functions that are not found in yeast. For example, in yeast cells there are relatively few microtubules, all of which terminate in the SPB, but in animal cells, many microtubules are not associated with the centrosome. At least some of these microtubules appear to arise by release from the centrosome (Keating et al., 1997), whereas others appear to arise de novo in the cytoplasm (Vorobjev et al., 1997). During mitosis in animal cells, γ -tubulin is found in the spindle, as well as at the centrosome (Lajoie et al., 1994), suggesting that the γ -tubulin complex is associated with microtubule ends outside of the centrosome. Plants represent the extreme case, in which there is no obvious organizing center and γ -tubulin colocalizes with microtubules in most areas of the cell (Liu et al., 1993, 1994). Perhaps the presence of a cytoplasmic \sim 32S γ -tubulin complex in animal cells reflects a requirement for microtubule nucleation and/or stabilization independent of the organizing center.

The yeast γ -tubulin complex does not contain α - and β -tubulin (Knop and Schiebel, 1997), and we did not find α - and β -tubulin to be components of the mammalian γ -tubulin complex. This is in contrast to the results from *Xenopus* eggs (Zheng et al., 1995) and sheep brain (Detraves et al., 1997), in which α - and β -tubulin were reported to be major components of the γ -tubulin complex. Whereas it is possible that the association of α - and β -tubulin with the γ -tubulin complex is weak and is lost during the procedure that we used for assessing the composition of the complex,

we find this unlikely for two reasons. First, the number of steps and amount of time between cell lysis and affinity purification (or precipitation) of the complex is far less in the simple immunoprecipitations that we have performed than in the full-scale purifications of the γ -tubulin complex (Zheng et al., 1995; Detraves et al., 1997). Second, we have never observed cosedimentation of α - and β -tubulin with the γ -tubulin complex in sucrose gradients under any condition tested.

Cytoplasmic γ -Tubulin Versus Centrosomal γ -Tubulin

Most of what is known about γ -tubulin comes from studies of the cytoplasmic γ -tubulin complex, yet the main site of γ -tubulin action is thought to be the centrosome. What is the relationship between the cytoplasmic and centrosomal forms of γ -tubulin? The forms seem to be interconvertible to some extent in that the cytoplasmic γ -tubulin complex in frog egg extracts is able to associate with frog sperm centrioles to create a functional centrosome (Felix et al., 1994; Stearns and Kirschner, 1994). It was not clear in these experiments whether the entire γ -tubulin complex or a subset of the complex associated with the centrosome. Moritz et al. (1995) reported the presence of rings in the pericentriolar material of centrosomes that both resembled the purified *Xenopus* γ -tubulin complex in solution (Zheng et al., 1995), and reacted with anti- γ -tubulin antibody, suggesting that the entire ring-shaped complex might be present. We have shown that both GCP2 and GCP3 are components of the cytoplasmic γ -tubulin complex, as well as centrosomal proteins. These results suggest that the cytoplasmic and centrosomal γ -tubulin complexes are similar, and possibly identical.

Possible Roles for GCP2 and GCP3 in the γ -Tubulin Complex

GCP2 and GCP3 colocalize and copurify with γ -tubulin in every condition that we examined. The fact that the GCP2/GCP3 pairing has been conserved from yeast to mammals indicates that there must be functional significance to having two related proteins in association with γ -tubulin. Based on two-hybrid interaction data, Knop et al. (1997) came to the conclusion that both Spc97p and Spc98p can interact with γ -tubulin. A simple hypothesis would be that the homology between GCP2 and GCP3 reflects a common interaction site with γ -tubulin. Thus, the yeast complex may represent the minimal complex of two molecules of γ -tubulin, and one each of Spc97p/GCP2 and Spc98p/GCP3. Knop et al. (1997) also showed that Spc97p and Spc98p are able to interact with each other. It is possible that Spc97p/GCP2 and Spc98p/GCP3 are required to bring the γ -tubulin molecules together in the proper orientation for interaction with microtubules, or for initiation of a microtubule. An interesting point is that if the minimal subunit contains two γ -tubulin molecules, then the total number of γ -tubulin molecules in the complex must be an even number. Given that microtubules nucleated from animal centrosomes typically have 13 protofilaments (Evans et al., 1985), this would seem to argue against a strict 1:1 γ -tubulin/tubulin heterodimer mechanism of microtubule nucleation, as proposed by Zheng et al. (1995), but is not incompatible with the protofilament model of Erickson

and Stoffer (1996). One way in which an even number of γ -tubulin subunits could interact with 13 protofilaments would be if there is a one-subunit overlap within the complex, which would be consistent with the "lock washer" appearance of the complex under certain conditions (Zheng et al. 1995). Clearly, understanding the detailed mechanism of microtubule nucleation will require additional experiments.

Results in yeast indicate that Spc97p and Spc98p mediate the attachment of γ -tubulin to the SPB by interaction with another SPB component, Spc110p (Knop and Schiebel, 1997). Spc110p is a coiled-coil protein that itself links the inner plaque, to which microtubules are attached, to the central plaque that is embedded in the nuclear envelope (Kilmartin et al., 1993). If GCP2 and GCP3 perform a similar role in the animal γ -tubulin complex, then one might expect to find an Spc110p-related protein in the centrosome. Interestingly, Tassin et al. (1997) have reported that a protein immunologically related to yeast Spc110p is present in vertebrate centrosomes.

One of the questions concerning the γ -tubulin complex is how its microtubule nucleation activity is regulated. There are substantial amounts of the cytoplasmic form of the γ -tubulin complex, yet the vast majority of microtubule nucleation appears to be limited to the centrosome. Thus, a possible role for γ -tubulin complex components would be to inhibit the interaction of the complex with tubulin or microtubules. Such an inhibitory protein might be a part of the γ -tubulin complex in the cytoplasm, but dissociate from the complex when it is attached to the centrosome, or when it binds to microtubules. Because GCP2 and GCP3 both localize to the centrosome and bind to microtubules along with γ -tubulin we consider it unlikely that they play an inhibitory role.

Many of the most important questions concerning γ -tubulin and the γ -tubulin complex will require in vitro reconstitution of all or part of the complex. With the identification of GCP2 and GCP3 as two conserved components of the complex, we have taken a step towards this goal and anticipate that study of these proteins and their interactions with γ -tubulin will help to elucidate the mechanisms behind the functions common to all microtubule organizing centers.

We thank L. Marschall and J. Carminati for comments on the manuscript.

This work was supported by grants to T. Stearns from the National Institute of General Medical Sciences of the National Institutes of Health, the American Cancer Society, and the Searle Foundation; and to S.M. Murphy from the American Cancer Society (PF-4198).

Received for publication 5 February 1998 and in revised form 23 March 1998.

References

- Akashi, T., Y. Yoon, and B.R. Oakley. 1997. Characterization of γ -tubulin complexes in *Aspergillus nidulans* and detection of putative γ -tubulin interacting proteins. *Cell Motil. Cytoskeleton*. 37:149–158.
- Blose, S.H., D.I. Meltzer, and J.R. Feramisco. 1984. 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* 98:847–858.
- Detraves, C., H. Mazarguil, M.I. Lajoie, M. Julian, M.B. Raynaud, and M. Wright. 1997. Protein complexes containing γ -tubulin are present in mammalian brain microtubule protein preparations. *Cell Motil. Cytoskeleton*. 36: 179–89.
- Dieckmann, C.L., and A. Tzagoloff. 1985. Assembly of the mitochondrial membrane system. *J. Biol. Chem.* 260:1513–1520.

- Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. Kilburn, W.-H. Lee, and S.J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7:555–569.
- Erickson, H.P., and D. Stoffer. 1996. Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γ tubulin. *J. Cell Biol.* 135:5–8.
- Evan, G.I., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell Biol.* 5:3610–3616.
- Evans, L., T. Mitchison, and M. Kirschner. 1985. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* 100:1185–1191.
- Felix, M.-A., C. Antony, M. Wright, and B. Maro. 1994. Centrosome assembly in vitro: Role of γ -tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* 124:19–31.
- Geissler, S., G. Pereira, A. Spang, M. Knop, S. Soues, J. Kilmartin, and E. Schiebel. 1996. The spindle pole body component Spc98p interacts with the γ -tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:3899–3911.
- Green, N., H. Alexander, A. Olson, S. Alexander, T.M. Shinnick, J.G. Sutcliffe, and R.A. Lerner. 1987. Immunogenic structure of the influenza virus hemagglutinin. *Cell.* 28:477–487.
- Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726 pp.
- Horio, T., and B.R. Oakley. 1994. Human γ -tubulin functions in fission yeast. *J. Cell Biol.* 126:1465–1473.
- Horio, T., S. Uzawa, M.K. Jung, B.R. Oakley, K. Tanaka, and M. Yanagida. 1991. The fission yeast γ -tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99:693–700.
- Hyman, A., D. Drechsel, D. Kellogg, S. Salser, K. Sawin, P. Steffen, L. Wordeman, and T. Mitchison. 1991. Preparation of modified tubulins. *Methods Enzymol.* 196:478–485.
- Keating, T., J. Peloquin, V. Rodionov, D. Momcilovic, and G. Borisy. 1997. Microtubule release from the centrosome. *Proc. Natl. Acad. Sci. USA.* 94:5078–5083.
- Kilmartin, J.V., S.L. Dyos, D. Kershaw, and J.T. Finch. 1993. A spacer protein in the *Saccharomyces cerevisiae* spindle pole body whose transcript is cell cycle-regulated. *J. Cell Biol.* 123:1175–1184.
- Knop, M., and E. Schiebel. 1997. Spc98p and Spc97p of the yeast γ -tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:6985–6995.
- Knop, M., G. Pereira, S. Geissler, K. Grein, and E. Schiebel. 1997. The spindle pole body component Spc97p interacts with the γ -tubulin of *Saccharomyces cerevisiae* and functions in microtubule organization and spindle pole body duplication. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:1550–1564.
- Lajoie, M.I., Y. Tollon, C. Detraves, M. Julian, A. Moisand, H.C. Gueth, A. Debec, P.I. Salles, A. Puget, H. Mazarguil, et al. 1994. Recruitment of anti-genic γ -tubulin during mitosis in animal cells: presence of γ -tubulin in the mitotic spindle. *J. Cell Sci.* 107:2825–2837.
- Li, Q., and H.C. Joshi. 1995. γ -Tubulin is a minus end-specific microtubule binding protein. *J. Cell Biol.* 131:207–214.
- Liu, B., J. Marc, H.C. Joshi, and B.A. Palevitz. 1993. A γ -tubulin related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. *J. Cell Sci.* 104:1217–1228.
- Liu, B., H.C. Joshi, T.J. Wilson, C.D. Silflow, B.A. Palevitz, and D.P. Snustad. 1994. γ -Tubulin in *Arabidopsis*: gene sequence, immunoblot, and immunofluorescence studies. *Plant Cell.* 6:303–314.
- Marschall, L., R. Jeng, J. Mulholland, and T. Stearns. 1996. Analysis of Tub4p, a yeast γ -tubulin-like protein: implications for microtubule organizing center function. *J. Cell Biol.* 134:443–454.
- Martin, M.A., S.A. Osmani, B.R. Oakley. 1997. The role of γ -tubulin in mitotic spindle formation and cell cycle progression in *Aspergillus nidulans*. *J. Cell Sci.* 110:623–633.
- Martin, O., R. Gunawardane, I. Iwamatsu, and Y. Zheng. 1998. Xgrip109: A γ -tubulin associated protein with an essential role in γ TuRC assembly and centrosome function. *J. Cell Biol.* 141:675–687.
- McLeod, M., M. Stein, and D. Beach. 1987. The product of the *mei3+* gene, expressed under the control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:729–736.
- Meads, T., and T.A. Schroer. 1995. Polarity and nucleation of microtubules in polarized epithelial cells. *Cell Motil. Cytoskeleton.* 32:273–288.
- Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. In *Guide to Yeast Genetics and Molecular Biology*, Vol. 194. C. Guthrie and G.R. Fink, editors. Academic Press, San Diego. 795–826.
- Morgan, D.O., J.M. Kaplan, J.M. Bishop, and H.E. Varmus. 1991. Production of p60^{c-src} by baculovirus expression and immunoaffinity purification. *Methods Enzymol.* 200:645–660.
- Moritz, M., M.B. Braunfeld, J.W. Sedat, B. Alberts, and D.A. Agard. 1995. Microtubule nucleation by γ -tubulin-containing rings in the centrosome. *Nature.* 378:638–640.
- Moudjou, M., N. Bordes, M. Paintrand, and M. Bornens. 1996. γ -Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.* 109:875–887.
- O'Reilly, D.R., L.K. Miller, and V.A. Luckow. 1992. *Baculovirus Expression Vectors: A Laboratory Manual*. W.H. Freeman & Co., New York. 347 pp.
- Oakley, C.E., and B.R. Oakley. 1989. Identification of γ -tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature.* 338:662–664.
- Oakley, B.R., C.E. Oakley, Y. Yoon, and M.K. Jung. 1990. γ -Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell.* 61:1289–1301.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schild, D., A.J. Brake, M.C. Kiefer, D. Young, and P.J. Barr. 1990. Cloning of three human multifunctional *de novo* purine biosynthetic genes by functional complementation of yeast mutations. *Proc. Natl. Acad. Sci. USA.* 87:2916–2920.
- Shu, H.-B., and H.C. Joshi. 1995. γ -Tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells. *J. Cell Biol.* 130:1137–1147.
- Sobel, S.G., and M. Snyder. 1995. A highly divergent γ -tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:1775–1788.
- Spang, A., S. Geissler, K. Grein, and E. Schiebel. 1996. γ -Tubulin-like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle formation. *J. Cell Biol.* 134:429–441.
- Stearns, T., and M. Kirschner. 1994. In vitro reconstitution of centrosome assembly and function: the central role of γ -tubulin. *Cell.* 76:623–638.
- Stearns, T., L. Evans, and M. Kirschner. 1991. γ -Tubulin is a highly conserved component of the centrosome. *Cell.* 65:825–836.
- Tassin, A.M., C. Celati, M. Paintrand, and M. Bornens. 1997. Identification of an Spc110p-related protein in vertebrates. *J. Cell Sci.* 110:2533–2545.
- Vogel, J.M., T. Stearns, C.L. Rieder, and R.E. Palazzo. 1997. Centrosomes isolated from *Spisula solidissima* oocytes contain rings and an unusual stoichiometric ratio of α/β tubulin. *J. Cell Biol.* 137:193–202.
- Vorobjev, I.A., T.M. Svitkina, and G.G. Borisy. 1997. Cytoplasmic assembly of microtubules in cultured cells. *J. Cell Sci.* 110:2635–2645.
- Zheng, Y., M.K. Jung, and B.R. Oakley. 1991. γ -Tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. *Cell.* 65:817–823.
- Zheng, Y., M.L. Wong, B. Alberts, and T. Mitchison. 1995. Nucleation of microtubule assembly by a γ -tubulin-containing ring complex. *Nature.* 378:578–583.