

γ -Tubulin Is a Minus End-specific Microtubule Binding Protein

Qingqin Li* and Harish C. Joshi*[‡]

*Graduate Program in Biochemistry and Molecular Biology and [‡]Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, Georgia 30322

Abstract. The role of microtubules in mediating chromosome segregation during mitosis is well-recognized. In addition, interphase cells depend upon a radial and uniform orientation of microtubules, which are intrinsically asymmetric polymers, for the directional transport of many cytoplasmic components and for the maintenance of the structural integrity of certain organelles. The slow growing minus ends of microtubules are linked to the centrosome ensuring extension of the fast growing plus ends toward the cell periphery. However, the molecular mechanism of this linkage is not clear. One hypothesis is that γ -tubulin, located at the cen-

trosome, binds to the minus ends of microtubules. To test this model, we synthesized radiolabeled γ -tubulin in vitro. We demonstrate here biochemically a specific, saturable, and tight ($K_d = 10^{-10}$ M) interaction of γ -tubulin and microtubule ends with a stoichiometry of 12.6 ± 4.9 molecules of γ -tubulin per microtubule. In addition, we designed an in vitro assay to visualize γ -tubulin at the minus ends of axonemal microtubules. These data show that γ -tubulin represents the first protein to bind microtubule minus ends and might be responsible for mediating the link between microtubules and the centrosome.

MANY cellular functions are dependent on the proper organization of microtubules. For example, in most eukaryotic cells the ordered distribution and transport of many cellular organelles including the Golgi apparatus, mitochondria, and the endoplasmic reticulum, is mediated by microtubules (Dabora and Sheetz, 1988; Kreis, 1990; Terasaki, 1990). Typically, microtubules are organized in a radial fashion due to their anchorage at a centrally located microtubule organizing center (MTOC)¹, represented by the centrosome in mammalian cells (Berns et al., 1977; Gould and Borisy, 1977; Kirschner, 1978; Pickett-Heaps et al., 1982). Within the characteristic radial array of interphase cells, these intrinsically polar polymers of α - and β -tubulin subunits are oriented such that the slow growing minus end is anchored at the centrosome ensuring the extension of the fast growing plus end toward the cell periphery (Heidemann and McIntosh, 1980). During mitosis, the dynamic array of the interphase microtubules reorganizes to construct a bipolar spindle array which ensures that one copy of each duplicated chromosome is correctly inherited by each of the nascent daughter cells (for a review see Brinkley, 1985). It is predicted that centrosomal components play the key role in microtubule organization.

A typical mammalian centrosome consists of a pair of centrioles constructed of microtubules and surrounded by an electron dense amorphous cloud of pericentriolar ma-

terial (PCM). Experimental and structural evidences demonstrate that microtubules terminate within the pericentriolar material which is responsible for the organizations of microtubule arrays during all phases of the cell cycle (Porter, 1966; McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Weisenberg and Rosenfeld, 1975; Peterson and Ris, 1976; Gould and Borisy, 1977; Telzer and Rosenbaum, 1979; Clayton et al., 1985). However, the molecular mechanisms by which the pericentriolar material mediates microtubule organization remain elusive. Emerging evidence suggests that a pericentriolar protein γ -tubulin might be involved (Oakley and Oakley, 1989, 1990; Stearns et al., 1991; Horio et al., 1991; Zheng et al., 1991; Joshi et al., 1992; Felix et al., 1994; Stearns and Kirschner, 1994). γ -Tubulin was first identified as an extragenic suppressor of a mutant β -tubulin allele in *Aspergillus nidulans* (Weil et al., 1986; Oakley and Oakley, 1989). cDNA sequence analysis and immunolocalization studies in various species reveal that γ -tubulin is a ubiquitous and highly conserved protein within the MTOCs in eukaryotic kingdom (Oakley and Oakley, 1989; Stearns et al., 1991; Horio et al., 1991; Zheng et al., 1991; Bass and Joshi, 1992; Joshi et al., 1992; Masuda, 1992; Fuchs et al., 1993; Liang and Heckmann, 1993; Luo and Perlin, 1993; Gueth-Hallonet et al., 1993; Liu et al., 1993; McDonald et al., 1993; Muresan et al., 1993; Palacios et al., 1993; Rizzolo and Joshi, 1993; Liu et al., 1994). Disruption of the γ -tubulin gene in fungi and *Drosophila* is lethal and leads to a pronounced depletion of microtubules (Oakley and Oakley, 1990; Horio et al., 1991; Sunkel et al., 1995). Mammalian cells fail to assemble mitotic spindles after microinjection of γ -tubulin antibody (Joshi et al., 1992), and depletion of γ -tubulin

Address correspondence to Harish C. Joshi, Emory University School of Medicine, Atlanta, GA 30322. Tel.: (404) 727-6273. Fax: (404) 727-6256.

1. *Abbreviations used in this paper:* MTOC, microtubule organizing center; PCM, pericentriolar material.

containing complex diminishes the ability of *Xenopus* egg extracts to support the sperm mediated formation of microtubule asters (Felix et al., 1994; Stearns and Kirschner, 1994). Although indirect, together these data indicate that γ -tubulin plays a key role in the organization of microtubules arrays, and predict that γ -tubulin binds to and nucleates microtubules at the centrosome. The test of this model requires study of biochemical interaction of γ -tubulin and microtubules. However, γ -tubulin is present in a low abundance 10^4 molecules per cell, less than 1% the level of either α - or β -tubulin, and purification has been difficult (Stearns et al., 1991). As an alternative, in this study, we used in vitro synthesized radiolabeled γ -tubulin to study the biochemical interaction of γ -tubulin with microtubules. Here we show that γ -tubulin tightly binds to microtubule ends in a saturable fashion in vitro, and the binding is specific to the minus ends of microtubules. We suggest that γ -tubulin organizes cytoplasmic microtubules by mediating the link between the slow growing minus ends of microtubules and the centrosome.

Materials and Methods

Vector Construction

To construct the plasmid pGEM-hyT, a 1568 base pairs full-length human γ -tubulin cDNA EcoRI fragment (from 24 bp upstream of the translation initiation site to 308 bp downstream of the stop codon) was excised from pBluescript SK-hyT vector (Zheng et al., 1991) and inserted at the EcoRI site of pGEM4 vector (Promega Corp., Madison, WI) downstream of T7 promoter. To construct the plasmid pGEM-hyT/Myc-tag, a EcoRI fragment containing hyT/Myc-tag was excised from phyT/Myc-tag (Shu and Joshi, 1995) and inserted at the EcoRI site downstream of T7 promoter.

Synthesis of Radiolabeled γ -Tubulin by In Vitro Transcription and Translation

pGEM-hyT and phyT/Myc-tag were linearized with SacI and the mRNA was synthesized in a 100 μ l reaction containing 5–10 μ g of the linearized DNA template, 40 mM Tris-HCl (pH 7.9), 6.0 mM MgCl₂, 2 mM Spermidine, 10 mM NaCl, 10 mM DTT, 100 units of RNasin (Promega), 100 μ g/ml BSA, 2.0 mM each of the nucleotide triphosphates (Pharmacia LKB Nuclear, Gaithersburg, MD) and 80 units of T7 RNA polymerase (Promega). Reaction was carried out at 35°C for 2 h. After RNA synthesis, the DNA template was digested by adding 5 units of RQ1-DNase (Promega) and incubating for 15 min at 37°C. Proteins were removed by phenol and chloroform extraction and the RNA was precipitated by ethanol, and dissolved in DEPC-treated water. The translation reaction was carried out at 30°C for 1 h in a 50- μ l reaction mixture containing 7 μ g of RNA, 35 μ l of rabbit reticulocyte lysate (Promega), 40 units of RNasin, 1 μ l of an amino acid mixture containing 1 mM each of the 19 amino acids (lacking methionine), and 60 μ Ci ³⁵S-methionine (specific activity 1000 Ci/m mol; Amersham Corp., Arlington Heights, IL). We also synthesized unlabeled γ -tubulin for microtubule binding competition assays by incubating the complete unlabeled amino acid mixture. After translation, the protein was subjected to two subsequent gel filtrations on Sephadex G-25 columns to separate unincorporated amino acids. The protein was either used immediately or stored at –80°C for future use.

In Vitro Microtubule Cosedimentation Assay

Tubulin proteins were prepared according to Vallee (1986) and Sloboda and Rosenbaum (1982). Rhodaminated tubulin proteins were prepared according to Hyman et al. (1991). Axonemes were prepared according to Witman et al. (1986) and Smith and Sale (1992). Protein concentration was assayed using BCA method (Smith et al., 1985). In vitro microtubule cosedimentation assay was carried out essentially as described in Yang et al. (1989). Microtubules were assembled in vitro by incubating PC-purified bovine brain tubulin or rhodaminated tubulin in PEM buffer (0.1 M Pipes, 2 mM EGTA, 1 mM MgSO₄) in the presence of 1 mM GTP at 37°C for 20 min. After this initial polymerization, the microtubule solution was

diluted with an appropriate volume of PEM buffer containing 1 mM GTP and 20 μ M Taxol and incubated at 37°C for additional 10 min. Radiolabeled γ -tubulin was spun at 10,000 g for 10 min in an ultracentrifuge (Airfuge; Beckman Instrs., Fullerton, CA) and the supernatant was used in the following microtubule cosedimentation assay. A constant amount of microtubules (5 μ l at a concentration of 0.4 μ g/ μ l) was incubated with increasing amounts of γ -tubulin (varying from 2 to 95 μ l) in a 100 μ l reaction volume supplemented with 1 mM GTP and 20 μ M Taxol at 37°C for 30 min. Microtubules were separated by centrifugation at 100,000 g through a 60% glycerol cushion for 10 min in an ultracentrifuge. The supernatant was mixed with 20 μ l of 5 \times SDS-sample buffer (Laemmli, 1970) and the microtubule pellet was dissolved into 20 μ l 1 \times SDS sample buffer and subjected to SDS-PAGE analysis. The gels were fluorographed, dried, and quantitated using PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA). Saturability of the binding reaction was analyzed by equilibrium binding curves, while the dissociation constant and Hill coefficient were determined by Scatchard plot analysis and Hill plot transformations of the primary data.

To generate more polymer ends while keeping the polymer mass constant, microtubules and axonemes were sheared by repetitively passing them through a 26-gauge hypodermic needle attached to a 1-ml tuberculin syringe (Becton, Dickinson & Co. Rutherford, NJ) and used in similar microtubule cosedimentation assays.

Assay for Microtubule Number Concentration Determination

Tetramethylrhodaminated tubulin proteins were prepared according to Hyman et al. (1991). Briefly, microtubules assembled from the phosphocellulose purified tubulin in 0.1 M Hepes, pH 8.6, 1 mM MgCl₂, 1 mM EGTA, 40% (vol/vol) glycerol were incubated with 1/10 volume of 100 mM tetramethylrhodamine succinimidyl ester at 37°C for 20 min. The reaction was stopped by diluting into low pH 2 \times BRB80 buffer (80 mM Pipes, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) containing 40% (vol/vol) glycerol. The microtubules were cycled (polymerization and depolymerization) twice after the labeling reaction and used in cosedimentation assays or frozen in liquid nitrogen for long term usage. The stoichiometry of labeling was determined by analyzing absorption spectra and was estimated to be 1.8 molecules tetramethylrhodamine per tubulin heterodimer for this preparation. To determine the concentration of microtubules or the axonemes used in the reaction, 5 μ l rhodaminated microtubules were mixed with 95 μ l green fluorescent labeled Covaspheres MX particles (Covalent Technology Corporation, Ann Arbor, MI) in PEM buffer supplemented with 1 mM GTP and 20 μ M Taxol. Three independent small aliquots (2 μ l) of the mixture were placed on a slide and visualized by fluorescence microscopy. Pictures of 36 random fields of the mixture under microscope were taken and the ratio of microtubules to particle was determined by counting each component in the negative directly. From the concentration of fluoresceinated beads that was measured in a hemocytometer, we calculated the number concentration of microtubules.

Quantitative Analysis of Autoradiograms

To characterize the interaction between γ -tubulin and microtubules, an accurate quantitation of the radiolabeled γ -tubulin was required. To circumvent the concern of a nonlinear response of x-ray films using conventional densitometry of the autoradiograms, we used a PhosphorImager which has a wide range of linear response. To ensure the linear response of the instrument, we first spotted a series dilution of ³⁵S-methionine solution on blotting paper and exposed it to the PhosphorImager screen. The optical densities obtained were then analyzed using ImageQuant software 3.3 (Molecular Dynamics). The amount of γ -tubulin in each binding reaction was analyzed by exposing the SDS-PAGE gels to the PhosphorImager screens, and the background was subtracted as recommended to account for the different background of different protein bands. Using the specificity of ³⁵S-methionine and data obtained from PhosphorImager analysis as well as microtubule number concentration, the stoichiometry of the binding reaction was deduced.

Visualization of γ -Tubulin Bound to the Minus End of Axonemal Microtubules

Flagellar axonemes were prepared from the flagella shed by *Chlamydomonas* cells induced with a pH shock (Witman, 1986; Smith and Sale, 1992; kindly provided by Dr. W. Sale and L. Fox, Emory University) and labeled with tetramethylrhodamine succinimidyl ester using the proce-

ture described by Hyman et al. (1991). In the γ -tubulin binding assay the labeled axonemes yielded a saturation binding curve similar to that of in vitro assembled microtubules. γ -Tubulin-bound axonemes or sheared axoneme fragments were attached to poly-L-lysine coated glass coverslips. The unbound sites on the coverslip were blocked by incubation with 2% casein in PEM buffer supplemented with 2 mM GTP at room temperature for 15 min. To visualize the myc-tag on the axoneme bound γ -tubulin molecules, the anti-myc-tag antibody was first attached to green fluorescent dye fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO) labeled *Staphylococcus aureus* particles in the following sequence: *S. aureus* particles were first sedimented by centrifugation from 100 μ l of cell suspension (Pansorbin; Calbiochem-Novabiochem, La Jolla, CA) and resuspended in 1 ml of 0.1 M sodium carbonate (pH 9.0). This suspension was then mixed with 50 μ l of 1 mg/ml FITC in anhydrous dimethyl sulfoxide and incubated for 4 h at room temperature. At the end of the incubation the labeled particles were pelleted in a microcentrifuge and washed five times each with 1 ml TBS. The fluorescent *S. aureus* particles were then incubated with the anti myc-tag antibody for 3 h at room temperature and washed five times with PEM buffer supplemented with 2 mM GTP to remove any excess unbound antibody. The glass coverslips containing the γ -tubulin bound axonemes or sheared axonemal fragments were then incubated at room temperature for 15 min with the suspension of the anti myc-tag containing FITC labeled *S. aureus* particles. The coverslips were washed 5 \times with PEM to remove the unbound particles, and then mounted in Aqua mount (Lerner Laboratories, New York). The coverslips were examined on a Zeiss Axiovert microscope with epifluorescence optics with a 100 \times Plan-neofluar objective (Carl Zeiss, Inc., Thornwood, NY) and a dual filter set that allows simultaneous visualization of both the rhodamine and FITC channels.

Results

Specific Interaction Between γ -Tubulin and Microtubules

If centrosomal γ -tubulin is directly involved in the organization of cellular microtubules, it must physically interact with microtubules. To test this hypothesis, we constructed a vector pGEM-hyT, in which the T7 promoter was used to drive a full length human γ -tubulin cDNA. With this vector, a single radioactive band of 49 kD, corresponding to the size of γ -tubulin, was produced in an in vitro transcription and translation reaction using 35 S-methionine (Fig. 1, A and B). We incubated the radiochemically tagged γ -tubulin with taxol-stabilized microtubules followed by sedimentation at 100,000 g for 10 min through a BRB80 buffer containing a 60% glycerol cushion and analysis by SDS-polyacrylamide gel electrophoresis (Fig. 1 C). Microtubules with bound γ -tubulin are collected in the pellet fraction. As shown in lane 1, γ -tubulin was not detected in the pellet fraction in the absence of microtubules. However, radiolabeled γ -tubulin was clearly detected in the pellet fraction when microtubules were added, suggesting a physical association of γ -tubulin with microtubules (Fig. 1 C, lane 2). A 20-fold excess of unlabeled γ -tubulin, also synthesized in vitro, efficiently competed with the binding of radioactive γ -tubulin (Fig. 1 C, lane 3). In addition, incorporation of biotinylated lysines into in vitro synthesized γ -tubulin abolished its ability to associate with microtubules (data not shown). These data suggest that γ -tubulin specifically interacts with microtubules.

Biochemical Characterization of the Interaction between γ -Tubulin and Microtubules

To determine if binding of γ -tubulin to microtubules is saturable, we examined the binding of increasing concentration of γ -tubulin with a constant mass of taxol-stabilized

microtubule assembled in vitro. After 30 min of incubation at 37°C, the free γ -tubulin and bound γ -tubulin were separated by sedimentation at 100,000 γ in an airfuge through BRB80 buffer containing a 60% glycerol cushion. The free γ -tubulin and bound γ -tubulin were resolved by SDS-PAGE and quantitated by phosphorimaging. Plotting bound γ -tubulin vs. total input γ -tubulin revealed a typical rectangular hyperbola, suggesting a saturable binding reaction between γ -tubulin and microtubules (Fig. 2 A). Scatchard plot analysis showed a high apparent affinity and minimum dissociation constant of 1.7×10^{-10} M and B_{\max} of 134 pM with respect to γ -tubulin concentration (Fig. 2 B). To determine if the interaction of γ -tubulin with microtubules is cooperative, we next performed a Hill plot analysis of the binding data. This analysis of the γ -tubulin interaction with microtubules resulted in a straight line with the value of n_H close to 1 (0.96; Fig 2 C) indicative of undetectable cooperativity of the binding reaction. The value of K_d from this analysis was 1.5×10^{-10} M, which was comparable to that of 1.7×10^{-10} M calculated from the Scatchard plot analysis. These data suggest that γ -tubulin binds tightly to microtubules in a saturable fashion.

The Interaction of γ -Tubulin Is Dependent on the Number Concentration of Microtubules

In vivo γ -tubulin is localized to the centrosome where the minus ends of the microtubules reside, suggesting that γ -tubulin might interact only with ends of microtubules in vitro. One prediction of this hypothesis is that for constant polymer mass, γ -tubulin binding will increase when more ends are present. To test this, we sheared a constant mass of microtubules by passing them through a hypodermic needle attached to a 1-ml tuberculin syringe. This procedure increased the number of microtubules as determined by direct visualization of fluorescent microtubules (Fig. 3). A typical experiment is shown in Fig. 4 in which the number of microtubules increased from 1.7×10^8 to 2.4×10^8 . Scatchard analyses of the binding reaction of γ -tubulin to both the sheared and unsheared microtubules reveal a pair of parallel lines with increased intercept at the X axis (Bound maximum), suggesting an increase in the amount of γ -tubulin after shearing and a constant apparent dissociation constant (Fig. 4). These data suggest that γ -tubulin interacts only with the ends of microtubules.

Determination of Microtubules Number Concentration and the Stoichiometry of Microtubule and γ -tubulin Interaction

Since γ -tubulin interacts with the ends of microtubules, it would be meaningful to determine the stoichiometry of maximal bound γ -tubulin versus number (molar) concentration of microtubule polymers, instead of simply using the protein mass concentration of microtubules. To measure precisely the number of microtubule in each reaction, we produced tetramethylrhodamine labeled microtubules (Fig. 3) that showed saturable binding curves identical to that of unlabeled microtubules as well as allowed the visualization of even a small fluorescent microtubule fragment used in the binding reaction. To determine precisely the concentration of individual microtubules, we mixed rhodaminated microtubules with a colloidal solution of fluorescent latex beads (1 μ m) (Fig. 3), the concentration of

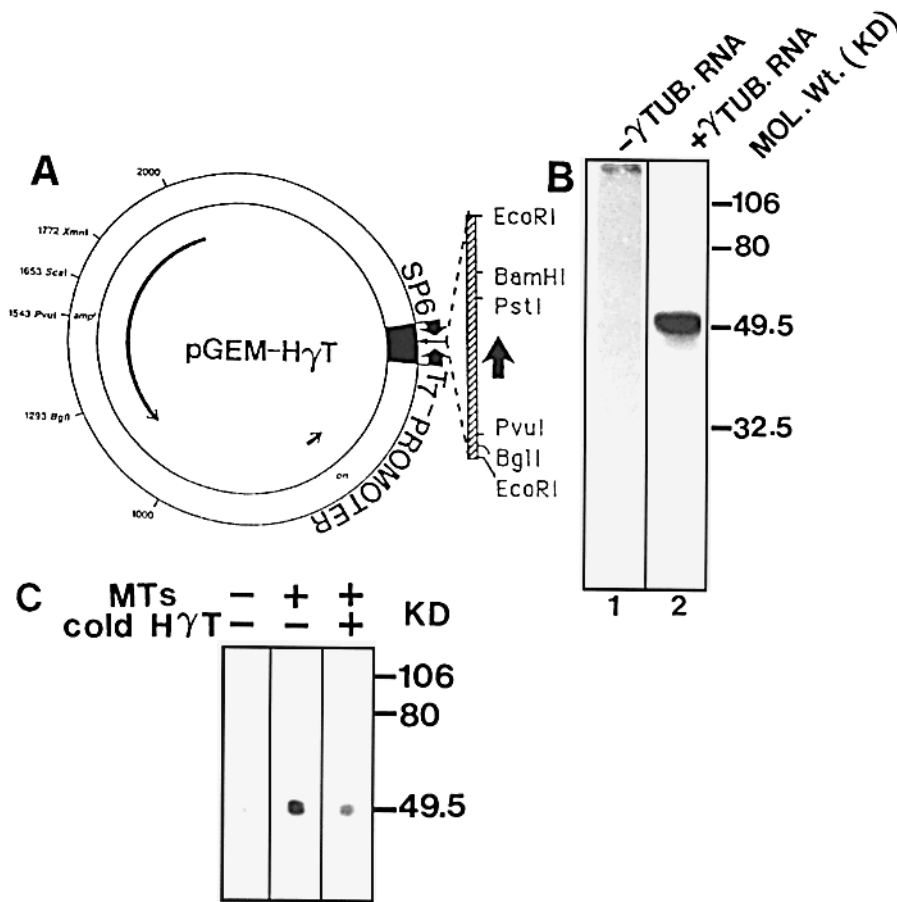


Figure 1. Synthesis of radiochemically pure mammalian γ -tubulin protein and its in vitro binding to microtubules. (A) Schematic of the plasmid construct, pGEM-h γ T, containing human γ -tubulin downstream of a T7 promoter used for in vitro transcription of γ -tubulin mRNA. (B) Autoradiogram of the in vitro synthesized 35 S-methionine labeled γ -tubulin in the presence (lane 2), and in the absence (lane 1) of γ -tubulin mRNA. A single radioactive band of the apparent molecular mass of 49 kD was detected in the presence of γ -tubulin mRNA. (C) Autoradiogram of the pellet fraction from microtubule cosedimentation assay using radiolabeled γ -tubulin without or with in vitro polymerized taxol-stabilized microtubules (lanes 1 and 2), and this binding reaction can be effectively competed out by the addition of a 20-fold excess of unlabeled γ -tubulin (lane 3).

which was also determined independently in a hemocytometer. The ratio of microtubules to beads from 36 random fields allowed us to calculate the number concentration of microtubules. From five independent experiments, maximal bound γ -tubulin was obtained from Scatchard plot analysis, and the number of microtubules was obtained as described above. Plotting bound (max) versus the number of microtubules from these experiments revealed a straight line with a slope (representation of stoichiometry) of 15.4 γ -tubulin molecules per microtubule (Fig. 5). The mean of individual values for stoichiometry is 12.6 ± 4.9 γ -tubulin molecules per microtubule.

γ -Tubulin Binds to Minus Ends of Axonemal Microtubules

The simplest model that emerges from the binding data described above is that γ -tubulin binds the ends of microtubules. To test further this hypothesis, we next sought to visualize the localization of molecules of bound γ -tubulin on microtubules. Our prediction was γ -tubulin would be located at the minus ends of microtubules. To do this, we initially used immunofluorescence labeling using our γ -tubulin antibody and the low light detection techniques. These initial efforts were unsuccessful primarily due to two reasons. First, the fluorescence signal generated by a few γ -tubulin molecules at the microtubule end may not be sufficient to detect over the background. Second, since our antibody was raised against a conserved region of γ -tubulin and the binding of this antibody to the centrosome disrupts the nu-

cleation of microtubules (Joshi et al., 1992). Therefore, this antibody may not be a suitable reagent for this experiment. To circumvent these problems, we made two modifications: first, we synthesized an epitope-tagged version of γ -tubulin in which a 14-amino acid myc-tag was fused in-frame at the extreme carboxyl terminus of the human γ -tubulin (Evan et al., 1985). The carboxy terminus of γ -tubulin was chosen because it is the most divergent domain and it contains variable number of amino acids in different species (for review see Joshi, 1993). In in vitro microtubule binding assays (such as illustrated in Fig. 2), the epitope-tagged γ -tubulin produced identical saturable curves compared with nontagged γ -tubulin and with both microtubules and with purified FITC labeled axonemes. The binding curve for sheared axonemes was also similar to that of taxol-stabilized microtubules. Second, we used rhodaminated axonemal microtubules in which the frayed plus ends of the microtubule bundle is clearly distinguishable from the unfrayed minus ends. The axonemes were incubated with the in vitro synthesized myc-tagged γ -tubulin and the γ -tubulin bound axonemes were diluted before their sparse attachment to a polylysine-coated glass coverslip (the concentrated axonemal suspension creates large clumps in this binding reaction). To meet the challenge of visualizing the few molecules of the myc-tagged γ -tubulin at the ends of bright red staining axonemal microtubules, we attached the anti-myc antibody on the FITC-labeled particles (*S. aureus* bacterial cells). Fig. 6 (A-F) shows those axonemes whose plus ends were frayed with the γ -tubulin bound at intact minus ends revealed by green

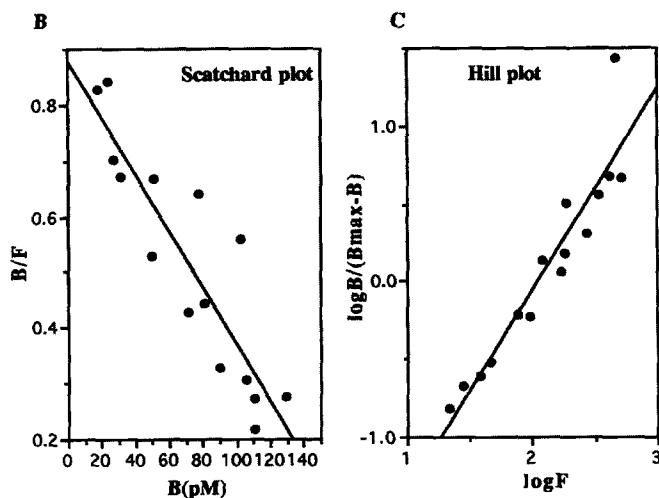


Figure 2. Characterization of the interaction between γ -tubulin and microtubules. A constant amount of taxol-stabilized microtubules was incubated with an increasing amount of radiolabeled γ -tubulin in a microtubule cosedimentation assay keeping the final taxol concentration constant as described in the Material and Methods. (A) Plotting the γ -tubulin bound (Y-axis) to taxol-stabilized microtubules assembled from purified bovine brain tubulin against free γ -tubulin (X-axis) reveals a typical rectangular hyperbola, suggesting a saturable interaction between γ -tubulin and microtubules. Insets show the autoradiograms of bound and free γ -tubulin that were used for the quantitative PhosphorImager analysis. B, Scatchard plot analysis of the binding data. A plot of bound (B) / free (F) vs. bound (B) yields a straight line with a K_d of 1.7×10^{-10} M and B_{max} of 134 pM. (C) Hill plot analysis. Plotting $\log(B/[B_{max}-B])$ vs. $\log F$ yields a straight line with a Hill coefficient (n_H) of 0.96 and K_d' of 1.5×10^{-10} M, suggesting a non-cooperative interaction.

antibody attached particles. Also shown are the more abundant normal unfrayed axonemes for comparison (Fig. 6, G and H). *S. aureus* bacterial cells alone did not bind to the ends of axonemes. Taken together, these results indicate γ -tubulin binds to the minus ends of microtubules.

Discussion

We have demonstrated an interaction of γ -tubulin with the minus ends of microtubules using γ -tubulin synthesized *in vitro* in the rabbit reticulocyte lysate translation system. This interaction is specific and saturable with an apparent dissociation constant of 10^{-10} M and a stoichiometry of 12.6 ± 4.9 molecules of γ -tubulin per microtubule. Our data are the first direct evidence that γ -tubulin specifically binds microtubules at minus ends. These results are

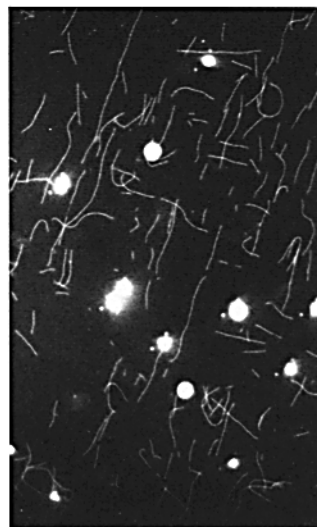


Figure 3. Assay for microtubules number concentration determination. The ratio of rhodaminated microtubules amidst fluorescent latex beads in 36 random fields was used to determine the number concentration of microtubules. A typical example is shown.

consistent with earlier genetic data indicating that γ -tubulin interacts with tubulin subunits. First, the isolation of the γ -tubulin gene as an extragenic suppressor of a temperature sensitive allele of the β -tubulin gene in *Aspergillus nidulans* suggested a physical interaction between β - and γ -tubulin (Weil et al., 1986; Oakley and Oakley, 1989). In addition, the combination of different mutant alleles of β -tubulin and γ -tubulin mutant (*mip A*) produced different synthetic phenotypes (Weil et al., 1986), suggesting an allele-specific interaction between β -tubulin and γ -tubulin. These studies combined with many other *in vivo* studies led to the hypothesis that γ -tubulin might be a microtubule binding protein. In this context, the results from present study provides a direct link for an endwise attachment of the minus ends of microtubules to the centrosomal protein, γ -tubulin.

We present three lines of direct evidence that γ -tubulin specifically binds microtubules at the ends. First, the binding is directly related to the number of free ends for a certain mass of microtubule polymers. This was demonstrated

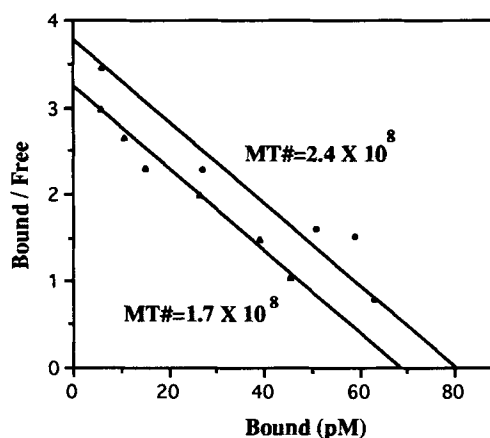


Figure 4. Maximal bound γ -tubulin to microtubules depends on number concentration of microtubules rather than mass concentration. A plot showing increasing microtubule ends generated by shearing of constant amount of microtubules by passing microtubule suspension through a 26-gauge hypodermic needle either 0 \times or 8 \times results in the progressively increased binding of γ -tubulin, while has no effect on the dissociation constant.

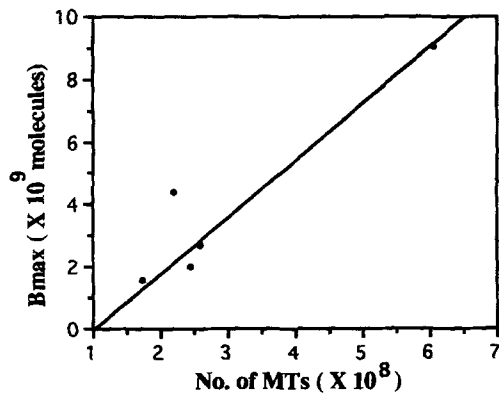


Figure 5. Determination of the stoichiometry of the binding interaction. In five independent experiments, the maximal amount of γ -tubulin bound to microtubules/axonemes and the number of microtubules/axonemes was determined as described in Fig. 2 *B* using Scatchard plot analysis and Fig. 4. A plot of maximal microtubule-bound γ -tubulin vs. number of microtubules reveals a stoichiometry (represented by the slope of the straight line) of 15.4 γ -tubulin per microtubule.

by shearing microtubules to short fragments, hence keeping the polymer mass constant, and demonstrating additional γ -tubulin binding. Second, the calculated binding stoichiometry of γ -tubulin to the polymerized α/β -tubulin dimer is variable ranging from a magnitude of 10^2 to 10^4 tubulin dimers per γ -tubulin molecule, while the constant binding stoichiometry of 12.6 ± 4.9 γ -tubulin per microtubule is consistent with binding at ends of microtubules rather than the overall lattice. Finally, immunofluorescence localization of γ -tubulin bound in vitro to the axonemal microtubules is restricted to the minus ends of uniformly oriented microtubules. These results, together with the previous experiments, implicate the centrosomal pro-

tein γ -tubulin as an organizer of microtubule arrays by linking the minus ends of microtubules to MTOCs.

Axonemal microtubule bundles naturally have uniform polarity. This polarity manifests itself in the structure of the isolated bundle at two ends, in that the plus ends of the bundle have a frayed appearance. While this feature of the axonemal microtubule bundle allowed us to designate the minus end as the γ -tubulin binding end of the microtubule, it also raised some new questions. The axonemes in vivo are continuous with the basal bodies that contain γ -tubulin and other proteins. Some procedures of axoneme preparation might be contaminated by basal bodies attached at their minus ends. One way to avoid this problem is to use flagella shed by *Chlamydomonas* during the physiologically induced excision process in which these cells actively excise the axonemal microtubules while retaining the basal bodies (see Witman, 1986, for a discussion of the axoneme isolation procedures). The axonemes prepared by this procedure extend their microtubules in vitro, at both ends, upon adding purified tubulin. Thus tubulin subunits at either end of the axoneme are thought to have exposed tubulin subunits. In addition, we found that the sheared axonemes show similar γ -tubulin binding curve analogous to taxol-stabilized microtubules. Thus, the new ends generated by the axoneme breaking show identical binding properties with the old ends. In summary, it is less likely that the binding of γ -tubulin to axoneme minus ends is mediated by other proteins, although there is still a formal possibility that γ -tubulin is binding to an axonemal component other than microtubules.

In 1992, Oakley and colleagues presented a model for the nucleation/attachment of microtubules at the centrosome. In this model, γ -tubulin binds to each protofilament by interacting physically with the β -tubulin subunit of an α - β heterodimer, thereby exposing α -tubulin at the distal plus ends of microtubule protofilaments (Oakley, 1992). This model is attractive because, in addition to de-

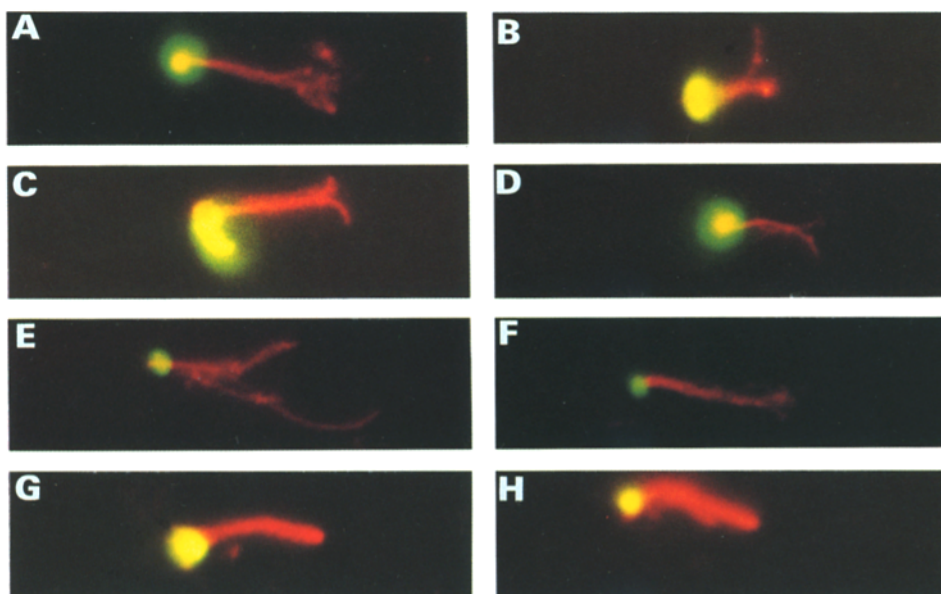


Figure 6. Visualization of epitope-tagged γ -tubulin bound at the minus ends of the axonemal microtubules. γ -Tubulin bound fluorescently labeled red axonemes were diluted to avoid aggregation and attached sparsely to glass coverslips. The bound γ -tubulin molecules were detected by an antibody to the epitope tag that was attached to fluorescently labeled green particles (*S. aureus* bacterial cells). As clearly seen from those axonemes that have their plus end frayed (*A-F*), the γ -tubulin visualized by green fluorescent particles is associated with the unfrayed minus ends of the microtubules. Also shown for comparison the more abundant unfrayed axonemes (*G* and *H*). FITC-labeled *S. aureus* particles alone did not bind to axonemes.

fining the centrosomal attachment of microtubule, it provides a molecular basis for orienting the fast growing plus ends of cellular microtubules away from the centrosome. Our studies indicate that γ -tubulin is a minus end specific microtubule binding protein, and hence, it might indeed provide a linkage for the attachment of the minus ends of microtubules to the centrosome. The question remains as to which one of the heterodimeric tubulin subunits is at the minus end and that one is at the plus end of a microtubule. This question has been addressed using two independent approaches. The first one has to do with the localization of the exchangeable GTP binding site. The NH₂-terminal domains of both α - and β -tubulin bind a GTP molecule. The α -tubulin-bound GTP is not exchangeable while the β -tubulin-bound GTP is exchangeable and becomes non-exchangeable upon polymerization (Jacobs et al., 1974; Geahlen and Haley, 1977; Zeeberg and Caplow, 1979; Zeeberg et al., 1980; Mandelkow et al., 1985; Nath and Himes, 1986; Mandelkow and Mandelkow, 1989). Recently, the exchangeable GTP site has been localized at the plus ends of microtubules suggesting the β -tubulin subunit may be at the plus end of the microtubule (Mitchison, 1993). However, in another set of experiments, a microtubule binding protein kinesin was used to decorate β -subunits followed by ultrastructural examination. These experiments revealed α -tubulin at the plus ends of microtubules and the kinesin bound β -tubulin at the minus end (Song and Mandelkow, 1995). The ultrastructural data are consistent with the original model of γ -tubulin microtubule interaction in which γ -tubulin might interact with β -tubulin of the tubulin heterodimer subunit at minus ends of microtubules.

Another interesting question is—what is the interaction interface between γ -tubulin and microtubule subunits? Does γ -tubulin bind to microtubules in a head-to-tail fashion similar to α - β dimers or does γ -tubulin bind to microtubules in a side-to-side fashion as microtubule protofilaments bind each other? Our studies suggest that the stoichiometry of γ -tubulin-microtubule interaction is 12.6 ± 4.9 molecules of γ -tubulin per microtubule. This would predict that the number of γ -tubulin molecules at the end of microtubule is close to the number of protofilaments in one microtubule such that each protofilament can bind one γ -tubulin at its minus end. Since a structural template of clearly distinguishable morphology has never been seen at the PCM embedded minus end of microtubule, it is possible that the microtubule binding interfaces of the terminal γ -tubulin subunits may resemble those of α -tubulin and β -tubulin and thus the linkage of γ -tubulin to microtubules may closely conform to the 13–15 protofilament lattice of microtubules during nucleation of microtubules by the centrosome.

During mitosis, tubulin subunits in the mitotic spindle are shown to flux toward the centrosomes (Mitchison, 1989). Thus any satisfactory model of microtubule nucleation and of linkage to the centrosome must explain the ability of the attached microtubule to lose subunits at minus end. In this context, it has been proposed that the centrosomal mechanisms of microtubule nucleation may be different from the mechanisms of microtubule attachment to the centrosome: after nucleation, microtubules may be released from the nucleation site to be recaptured by plus

end directed kinesin like motor molecules (Sawin et al., 1992). It is thus possible that γ -tubulin interacts with microtubules only during the nucleation step and is released afterwards in vivo. In any event, being a minus end binding protein, γ -tubulin is a good candidate to participate in the microtubule nucleation and/or attachment by the centrosome.

We thank Dr. B. Oakley of Ohio State University for providing γ -tubulin cDNA and for discussion; Dr. W. Sale, Dr. D. Howard, L. Fox and J. Habermacher of Emory University and T. Mitchison of University of California at San Francisco for technical help and discussion; Dr. E. Morgan for help with the quantitation by laser scanning densitometer; Drs. T. Folks and S. Bartel of CDC and D. Horon of Molecular Dynamics for help with PhosphorImager; Dr. R. Gunn for helping with the scatchard analysis; D. Aiken of Carl Ziess for his help in setting up the color charge coupled device (CCD); Drs. L. Coluccio, D. Reines, T. Pollard, and G. Boris for discussions; and C. Hayden and B. Hayden of Prophotography and C. Bogle of the Medical Illustration department at Emory University for help with the photography; and E. Chávez for help with the manuscript. Taxol was generously provided by the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

This work was supported partially by grants to H. C. Joshi from the National Institutes of Health, the American Cancer Society, the American Heart Association, and the University Research Committee at Emory University School of Medicine.

Received for publication 19 May 1995 and in revised form 28 June 1995.

References

- Baas, P. W., and H. C. Joshi. 1992. γ Tubulin distribution in the neuron: implications for the origin of neuritic microtubules. *J. Cell Biol.* 119:171–178.
- Berns, W., J. B. Rattner, S. Brenner, and S. Meredith. 1977. The role of the centriolar region in animal cell mitosis. *J. Cell Biol.* 72:351–367.
- Brinkley, B. R. 1985. Microtubule organizing centers. *Annu. Rev. Cell Biol.* 1: 145–172.
- Clayton, L., C. M. Black, and C. M. Lloyd. 1985. Microtubule nucleating sites in higher plant identified by an autoantibody against pericentriolar material. *J. Cell Biol.* 101:319–324.
- Dabora, S. L., and M. P. Sheetz. 1988. The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell.* 54:27–35.
- Doxsey, S. J., P. Stein, L. Evans, P. D. Calarco, and M. W. Kirschner. 1994. Pericentrin, a highly conserved centrosome protein involved in microtubule organization. *Cell.* 76:639–650.
- 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.
- Felix, G. W., 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.
- Fuchs, U., B. Moepps, H. P. Maucher, H. Schraudolph. 1993. Isolation, characterization and sequence of a cDNA encoding gamma-tubulin protein from the fern *Anemia phyllitidis* L. *Sw. Plant Mol. Biol.* 23:595–603.
- Geahlen, R. L., and B. E. Haley. 1977. Interactions of a photoaffinity analog of GTP with the proteins of microtubules. *Proc. Natl. Acad. Sci. USA.* 74:4375–4377.
- Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* 73:601–615.
- Gueth-Hallonet, C., C. Antony, J. Aghion, A. Santa-Maria, I. Lajoie-Mazenc, M. Wright, B. Maro. 1993. γ -Tubulin is present in acentriolar MTOCs during early mouse development. *J. Cell Sci.* 105:157–166.
- Heidemann, S. R., and J. R. McIntosh. 1980. Visualization of the structure polarity of microtubules. *Nature (Lond.)* 286:517–519.
- 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. Salsler, K. Sawin, P. Steffen, L. Wordeman, and T. Mitchison. 1991. Preparation of modified tubulins. *Methods Enzymol.* 196:478–485.
- Jacobs, M., H. Smith, and E. W. Taylor. 1974. Tubulin: nucleotide binding and enzymatic activity. *J. Mol. Biol.* 89:455–468.
- Joshi, H. C. 1993. The hub of cellular microtubule assemblies. *Bioessays.* 15: 637–643.
- Joshi, H. C. 1994. Microtubule organizing centers and γ -tubulin. *Current Opin. Cell Biol.* 6:55–62.

- Joshi, H. C., M. J. Palacios, L. McNamara, and D. W. Cleveland. 1992. γ Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature (Lond.)*. 356:80–83.
- Kirschner, M. W. 1978. Microtubule assembly and nucleation. *Int. Rev. Cytol.* 54:1–71.
- Kreis, T. E. 1990. Role of microtubules in the organization of the Golgi apparatus. *Cell Motil. Cytoskeleton.* 15:67–70.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
- Liang, A., and K. Heckmann. 1993. The macronuclear γ -tubulin-encoding gene of *Euplotes octocarinatus* contains two introns and an in-frame TGA. *Gene (Amst.)*. 136:319–322.
- Liu, B., J. Marc, H. C. Joshi, and B. A. Palevitz. 1993. A γ -tubulin related protein associated with the microtubule arrays of higher plant in a cell cycle dependent manner. *J. Cell Sci.* 104:1217–1228.
- Liu, B., H. C. Joshi, T. J. Wilson, C. D. Sifflow, B. A. Palevitz, D. P. Snustad. 1994. γ -Tubulin in *Arabidopsis*: gene sequence, immunoblot, and immunofluorescence studies. *Plant Cell.* 6:303–314.
- Luo, H., and M. H. Perlman. 1993. The γ -tubulin-encoding gene from the basidiomycete fungus, *Ustilago violacea*, has a long 5'-untranslated region. *Gene (Amst.)*. 137:187–194.
- Mandelkow, E., and E.-M. Mandelkow. 1989. Tubulin, microtubules, and oligomers: molecular structure and implications for assembly. In *Cell Movement*, Vol. 2: Kinesin, Dynein, and Microtubule Dynamics. Warner, F. D., and J. R. McIntosh, editors. Allen R. Liss, Inc., NY. 23–45.
- Mandelkow, E.-M., M. Herrmann, and U. Ruhl. 1985. Tubulin domains probed by limited proteolysis and subunit-specific antibodies. *J. Mol. Biol.* 185:311–327.
- Marchesi, V. T., and N. Ngo. 1993. *In vitro* assembly of multiprotein complexes containing α , β , and γ tubulin, heat shock protein HSP70, and elongation factor 1 α . *Proc. Natl. Acad. Sci. USA.* 90:3028–3032.
- Masuda, H., M. Sevik, and W. Z. Cande. 1992. *In vitro* microtubule-nucleating activity of spindle pole bodies in fission yeast *Schizosaccharomyces pombe*: cell cycle-dependent activation in *Xenopus* cell-free extracts. *J. Cell Biol.* 117:1055–1066.
- McDonald, A. R., B. Liu, H. C. Joshi, and B. A. Palevitz. 1993. γ -tubulin is associated with a cortical-microtubule-organizing zone in the developing guard cells of *Allium cepa* L. *Planta.* 191:357–361.
- McGill, M., and B. R. Brinkley. 1975. Human chromosomes and centrioles are nucleating sites for the *in vitro* assembly of microtubules from bovine brain tubulin. *J. Cell Biol.* 67:188–199.
- Melki, R., I. E. Vainberg, R. L. Chow, and N. Cowan. 1993. Charperonin-mediated folding of vertebrate actin-related protein and γ -tubulin. *J. Cell Biol.* 122:1301–1310.
- Mitchison, T. J. 1989. Polewards microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. *J. Cell Biol.* 109:637–652.
- Mitchison, T. J. 1993. Localization of an exchangeable GTP binding site at the plus end of microtubules. *Science (Wash. DC)*. 261:1044–1047.
- Muresan, V., H. C. Joshi, and J. Besharse. 1993. γ -Tubulin in differentiated cell types: localization in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia. *J. Cell Sci.* 104:1229–1237.
- Nath, J. P., and R. H. Himes. 1986. Localization of the exchangeable nucleotide binding domain in beta-tubulin. *Biochem. Biophys. Res. Commun.* 135:1135–1143.
- Oakley, B. R. 1992. Gamma-tubulin: the microtubule organizer? *Trends Cell Biol.* 2:1–5.
- Oakley, B. R., and C. E. Oakley. 1990. Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell.* 61:1289–1301.
- Oakley, C. E., and B. R. Oakley. 1989. Identification of gamma tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature (Lond.)*. 338:662–664.
- Palacios, M. J., H. C. Joshi, C. Simerly, and G. Schatten. 1993. Dynamic reorganization of γ -tubulin during mouse fertilization and early development. *J. Cell Sci.* 104:383–389.
- Peterson, J. B., and H. Ris. 1976. Electromicroscope study of the spindle and chromosome movement in the yeast *S. cerevisiae*. *J. Cell. Sci.* 22: 219–242.
- Pickett-Heaps, J. D., D. W. Tippet, and K. R. Porter. 1982. Rethinking mitosis. *Cell.* 29:729–744.
- Porter, K. R. 1966. Principles of biomolecular organization. G. E. Wolstenholme and M. O'Connor, editors. Little and Brown, Boston, MA. 308–345.
- Raff, J. W., D. R. Kellogg, and B. Albert. 1993. *Drosophila* γ -tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823–835.
- Rizzolo, L. J., and H. C. Joshi. 1993. Apical orientation of the microtubule organizing center and associated γ -tubulin during the polarization of the retinal pigment epithelium *in vivo*. *Developmental Biol.* 157:147–156.
- Sawin, K. E., K. LeGuellec, M. Philippe, and T. J. Mitchison. 1992. Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature (Lond.)*. 359:540–543.
- 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.* 130:1137–1148.
- Sloboda, R. D., and J. L. Rosenbaum. 1982. Purification and assay of microtubule-associated proteins (MAPs). *Methods Enzymol.* 85:409–416.
- Smith, E. F., and W. S. Sale. 1992. Structural and functional reconstitution of inner dynein arms in *Chlamydomonas* flagellar axonemes. *J. Cell Biol.* 117: 573–581.
- Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, and M. D. Provenzano. 1985. Measurement of protein using bicinchonic acid. *Anal. Biochem.* 150:76–85.
- Snyder, J. A., and J. R. McIntosh. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* 67: 744–760.
- Song, Y.-H., and E. Mandelkow. 1995. The anatomy of flagellar microtubules: polarity, seam, junction, and lattice. *J. Cell Biol.* 128:81–94.
- Stearns, T., and M. W. Kirschner. 1994. *In vitro* reconstitution of centrosome assembly and function: the central role of γ tubulin. *Cell.* 76:623–637.
- Stearns, T., L. Evans, and M. W. Kirschner. 1991. γ Tubulin is a highly conserved component of the centrosome. *Cell.* 65:825–836.
- Sunkel, C. E., R. Gomes, P. Sampaio, J. Perdigao, and C. Gonzalez. 1995. γ -Tubulin is required for the structure and function of the microtubule organizing center in *Drosophila* neuroblasts. *EMBO J.* 14:28–36.
- Telzer, B. R., and J. C. Rosenbaum. 1979. Cell cycle-dependent, *in vitro* assembly of microtubules onto the pericentriolar material of HeLa cells. *J. Cell Biol.* 35:159–179.
- Terasaki, M. 1990. Recent progress on structural interactions of the endoplasmic reticulum. *Cell Motil. Cytoskeleton.* 15:71–75.
- Vallee, R. B. 1986. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins and MAP fragments. *Methods Enzymol.* 134:89–104.
- Weil, C. F., C. E. Oakley, and B. R. Oakley. 1986. Isolation of *mip* (microtubule-interacting protein) mutations of *Aspergillus nidulans*. *Mol. Cell. Biol.* 6:2963–2968.
- Weisenberg, R. C., and A. C. Rosenfeld. 1975. *In vitro* polymerization of microtubules into asters and spindles in homogenates of surf clam eggs. *J. Cell Biol.* 64:146–158.
- Witman, G. B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods Enzymol.* 134:280–290.
- Yang, J. T., R. A. Laymon, and L. S. Goldstein. 1989. A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analyses. *Cell.* 56:879–889.
- Zeeberg, B., and M. Caplow. 1979. Determination of free and bound microtubular protein and guanine nucleotide under equilibrium conditions. *Biochemistry.* 18:3880–3886.
- Zeeberg, B., J. Cheek, and M. Caplow. 1980. Exchange of tubulin dimer into rings in microtubule assembly—disassembly. *Biochemistry.* 19: 5078–5086.
- 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.