Characterization of Two Related *Drosophila* γ-tubulin Complexes that Differ in Their Ability to Nucleate Microtubules

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Abstract. γ-tubulin exists in two related complexes in *Drosophila* embryo extracts (Moritz, M., Y. Zheng, B.M. Alberts, and K. Oegema. 1998. *J. Cell Biol.* 142:1–12). Here, we report the purification and characterization of both complexes that we name γ-tubulin small complex (γTuSC; ~280,000 D) and *Drosophila* γTuRC (~2,200,000 D). In addition to γ-tubulin, the γTuSC contains Dgrip84 and Dgrip91, two proteins homologous to the Spc97/98p protein family. The γTuSC is a structural subunit of the γTuRC, a larger complex containing about six additional polypeptides. Like the γTuRC isolated from *Xenopus* egg extracts (Zheng, Y., M.L. Wong, B. Alberts, and T. Mitchison. 1995. *Nature.* 378:578–583), the *Drosophila* γTuRC can nucleate microtubules in vitro and has an open ring structure with a diameter of 25 nm. Cryo-electron microscopy reveals a modular structure with ~13 radially arranged structural repeats. The γTuSC also nucleates microtubules, but much less efficiently than the γTuRC, suggesting that assembly into a larger complex enhances nucleating activity. Analysis of the nucleotide content of the γTuSC reveals that γ-tubulin binds preferentially to GDP over GTP, rendering γ-tubulin an unusual member of the tubulin superfamily.

Key words: centrosome • cytoskeleton • nucleation • GTP • GDP

THE microtubule (MT)[1] cytoskeleton is essential for cell division and organization of the interphase cytoplasm. These functions are orchestrated by diverse and highly dynamic MT arrays generated by a variety of mechanisms including regulation of the polymerization dynamics of MTs, of proteins that interact with and organize MTs, and of MT nucleation (Desai and Mitchison, 1997). The latter mechanism is possible because the spontaneous nucleation of new tubulin polymers is kinetically limiting, both in vitro when the polymerization of pure tubulin is initiated, and in vivo (Alberts et al., 1994).

Evidence for a kinetic barrier to MT nucleation in vivo comes from analysis of repolymerization of MTs after cold treatment or treatment with anti-MT agents. In many animal cells, regrowth initiates from the pericentriolar material (PCM) that surrounds the centrioles (Frankel, 1976; Osborn and Weber, 1976; Keryer et al., 1984; Meads and Schroer, 1995), demonstrating that the PCM promotes MT nucleation. A major breakthrough in defining the molecular basis of the MT-nucleating activity of the PCM was the discovery of γ-tubulin (Oakley and Oakley, 1989). γ-tubulin is a member of the tubulin superfamily that localizes to MT organizing centers and is found in all eukaryotes (reviewed in Joshi, 1994; Pereira and Schiebel, 1997). Genetic studies have demonstrated that γ-tubulin is required for normal cytoplasmic and spindle MT formation in *Aspergillus nidulans* (Martin et al., 1997; Oakley et al., 1990), *Schizosaccharomyces pombe* (Horio et al., 1991), *Drosophila melanogaster* (Sunkel et al., 1995), and *Saccharomyces cerevisiae* (Sobel and Snyder, 1995; Marschall et al., 1996; Spang et al., 1996). Antibody inhibition experiments in vertebrates have also implicated γ-tubulin in MT nucleation by the centrosome (Joshi et al., 1992; Felix et al., 1994).

In higher eukaryotes, soluble γ-tubulin exists primarily in a large complex (between 25 and 32 S; Stearns and Kirsch-

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1. *Abbreviations used in this paper:* Dgrip, *Drosophila* gamma ring protein; γTuRC, γ-tubulin ring complex; γTuSC, γ-tubulin small complex; MT, microtubule; PCM, pericentriolar material; PEG, polyethylene glycol; Sc γTuSC, *Saccharomyces cerevisiae* 6 S complex.

ner, 1994; Meads and Schroer, 1995; Zheng et al., 1995; Detraves et al., 1997; Moritz et al., 1998; Murphy et al., 1998). Recently, this complex was purified from *Xenopus* egg extracts and shown to nucleate MTs in vitro (Zheng et al., 1995). This complex, called the γTuRC (γ-tubulin ring complex), consists of about eight proteins in addition to γ-tubulin and has the appearance of an open ring with approximately the same diameter as a MT (Zheng et al., 1995). Rings of this diameter have also been observed in the PCM of centrosomes isolated from *Drosophila* embryos (Moritz et al., 1995a) and the surf clam, *Spisula* (Vogel et al., 1997). In *Drosophila*, immunoelectron microscopy has confirmed the presence of clusters of γ-tubulin in the ring structures and at the base of MTs nucleated by the PCM (Moritz et al., 1995b). Cumulatively, these results suggest that the γTuRC is a highly conserved structure responsible for the MT-nucleating activity of the PCM.

The γ-tubulin in *S. cerevisiae* is the most divergent of all γ-tubulins. It is only ~35–40% identical to the other known γ-tubulins, all of which are at least 65% identical to each other (Marschall et al., 1996). In *S. cerevisiae*, the only known soluble γ-tubulin–containing complex is ~6 S and contains three proteins: γ-tubulin, and two related proteins, Spc97p and Spc98p (Geissler et al., 1996; Knop et al., 1997; Knop and Schiebel, 1997). Immunoprecipitation experiments with tagged proteins suggest that the *S. cerevisiae* complex contains one molecule of Spc97p, one molecule of Spc98p, and two or more molecules of γ-tubulin (Knop et al., 1997; Knop and Schiebel, 1997). The yeast γ-tubulin 6 S complex is thought to be anchored to the cytoplasmic side of the spindle pole body through the interaction of Spc97p and Spc98p with Spc72p (Knop and Schiebel, 1998), and to the nuclear side of the spindle pole body through interaction with the NH_2 terminus of Spc110p (Knop and Schiebel, 1997). To date, in vitro MT-nucleating activity for the yeast complex has not been demonstrated. Therefore, it remains unclear whether the yeast γ-tubulin complex nucleates MTs directly, or whether it assembles into a larger, perhaps γTuRC-like structure at the spindle pole body. Interestingly, homologues of Spc97p and Spc98p in humans (hGCP2 and hGCP3/HsSpc98; Murphy et al., 1998; Tassin et al., 1998) and in *Xenopus* (Xgrip109; Martin et al., 1998) colocalize with γ-tubulin at the centrosome and cosediment with γ-tubulin on sucrose gradients, indicating that they are components of the large γ-tubulin–containing complexes present in these organisms.

Understanding the role of γ-tubulin in MT nucleation is a challenging endeavor. Low cellular concentrations make purification from native sources difficult, and the complexity of the protein complexes that contain γ-tubulin limits expression-based studies. Analysis of MT nucleation is further complicated by the following: the complex structure of the MT lattice (Wade and Chretien, 1993), the large number of tubulin molecules potentially involved in the formation of a nucleus (Voter and Erickson, 1984; Fygenson et al., 1995), and the potential role of β-tubulin GTP hydrolysis in suppressing nucleation (Hyman et al., 1992). This difficulty is reflected by the fact that the mechanism of spontaneous nucleation of purified tubulin remains poorly understood (Voter and Erickson, 1984; Fygenson et al., 1995).

Central to understanding the mechanism of MT nucleation by γ-tubulin–containing complexes will be to understand the relationship between γ-tubulin and other members of the tubulin superfamily. One important aspect of this relationship is the nature of the contacts γ-tubulin makes with itself and with α- or β-tubulin. A second important aspect is how γ-tubulin compares to other members of the tubulin family in its ability to bind and hydrolyze GTP. If γ-tubulin binds a guanine nucleotide, it will be important to determine whether nucleotide exchange and hydrolysis contribute to its ability to assemble, disassemble, nucleate, or release MTs, or whether the bound nucleotide has a structural role, as is the case for α-tubulin.

In this paper, we begin to address the functional organization of the γTuRC by purifying and analyzing γ-tubulin–containing complexes from *Drosophila* embryo extracts. In *Drosophila*, there are two related γ-tubulin–containing complexes. The larger complex can be collapsed into the smaller complex by treatment with high salt. This condensation suggests that the small complex is a structural subunit of the large complex (Moritz et al., 1998). We purify both complexes and show that the large *Drosophila* complex nucleates MTs much more potently than the small complex. We also show that, in contrast to α- and β-tubulin which preferentially bind GTP, γ-tubulin in the small complex preferentially binds GDP.

Materials and Methods

Buffers and Reagents

HB: 50 mM K-Hepes, pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol (β-ME) and protease inhibitor stock (1:200 final dilution; see below). HB100: HB plus 100 mM NaCl; HB200: HB plus 200 mM NaCl; and HB500: HB plus 500 mM NaCl. EB200: HB200 plus 100 μM GTP and 1 mg/ml DrosC17 peptide; EB500: HB500 plus 100 μM GTP and 1 mg/ml DrosC17 peptide; HB block: 50 mM K-Hepes, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mg/ml bovine serum albumin (fraction V; Sigma Chemical Co.). Homogenization buffer: HB100 plus 10% glycerol, 1 mM PMSF and protease inhibitor stock (1:100 final dilution). Protease inhibitor stock: 10 mM benzamidine-HCl, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A in ethanol. LPC: 10 mg/ml leupeptin, 10 mg/ml pepstatin A, and 10 mg/ml chymostatin dissolved in DMSO. Mounting medium: 20 mM Tris-Cl, pH 9.0, 90% glycerol, and 0.1% *p*-phenylenediamine. BRB80: 80 mM K-PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA; 4× sample buffer: 250 mM Tris, pH 6.8, 12% SDS wt/vol, 20% β-ME vol/vol, and 40% glycerol vol/vol. GTP stock: 100 mM GTP (Boehringer Mannheim Corp.). Tubulin was purified from the bovine brain and labeled with tetramethylrhodamine as described (http://skye.med.harvard.edu). *Drosophila* embryo extract was prepared by homogenizing 0–3.5-h *Drosophila* embryos in homogenization buffer as described (Moritz et al., 1998). Clarified extract was prepared by centrifugation of crude extract for 10 min at 15,000 rpm (SS34 rotor; Sorvall) at 4°C. The supernatant was transferred to a new tube, and centrifuged a second time at 50,000 rpm in a rotor (50.2 Ti or SW55; Beckman) for 1 h at 4°C.

Sucrose Gradient Sedimentation and Gel Filtration Chromatography

Sucrose gradients (5–20 or 5–40%) were poured as step gradients (five steps of equal volume) in HB containing 100 or 500 mM NaCl plus nucleotide and allowed to diffuse into continuous gradients. Gradients were fractionated from the top by hand with cutoff pipet tips. Fractions from standards gradients run in parallel were separated by 10% PAGE and stained with Coomassie blue. Gels were scanned, band intensities were quantitated (Adobe Photoshop; Adobe Systems Inc.) and peak fractions were assigned (Kaleidagraph Synergy Software Inc.). Standard curves of peak fraction versus sedimentation coefficient ($S_{20,w}$) were used to estimate S values of protein complexes.

Gel filtration chromatography was carried out on a column (Superose-6; Pharmacia Biotech Sverige) in HB plus 100 μM GTP, and 100 or 500 mM NaCl as indicated. The column was calibrated with standards of known Stokes radii. Molecular weights and Stokes radii of protein complexes were estimated as described (Siegel and Monty, 1966). Fractions were separated by SDS-PAGE on 10% gels and γ-tubulin was detected by Western blotting.

Immunoisolation of γ-tubulin–containing Complexes from Drosophila Embryo Extract

PEG (polyethylene glycol P-2139; average mol wt = 8,000; Sigma Chemical Co.) was added to a final concentration of 2% (from a 30% stock in HB100) to clarified *Drosophila* embryo extract from 20-g embryos. The mixture was incubated on ice for 20 min, spun at 17,000 rpm for 10 min in a SS34 rotor and the supernatant was discarded. The pellets were resuspended in 20 ml of HB200 plus 0.05% NP-40, and 100 μM GTP by gentle Dounce homogenization and clarified at 35,000 rpm for 30 min in a 50.2 Ti rotor. γ-tubulin complexes were immunoprecipitated from the supernatant by adding 190 μg of DrosC17 antibody and incubating at 4°C for 1 h with gentle rotation. The immunoprecipitate was collected by slowly (over 1 h) pumping the antibody-extract mixture over a 350-μl column of protein A–agarose (GIBCO/BRL) in a disposable Bio-spin column housing (Bio-Rad). The column was washed with 15 ml of HB200 plus 0.05% NP-40 and 100 μM GTP, and 15 ml of the same buffer without NP-40. 400 μl of EB200 was loaded onto the column and the column was sealed with parafilm and incubated for 16–18 h at 4°C. γ-tubulin complexes were collected by loading an additional 400 μl of EB200 onto the column and collecting the flow through. For the sucrose gradient fractionation described in Fig. 2, 150 μl of isolated complexes was loaded onto a 2.1-ml 5–40% sucrose gradient, poured in HB100 plus 100 μM GTP, and sedimented at 50,000 rpm for 4 h in an TLS55 rotor at 4°C.

Electron Microscopy

Negative stain electron microscopy of peptide-eluted complexes and sucrose gradient purified γTuRC was performed as described (Zheng et al., 1995), except that grids of sucrose gradient fractions were rinsed with water before staining. For cryo-electron microscopy fresh peptide-eluted γ-tubulin complexes were applied to glow discharged, holey carbon films supported on copper EM grids. Excess liquid was removed by blotting and the resulting thin film was rapidly frozen by plunging into liquid ethane slush. Frozen grids were stored in liquid nitrogen. Grids were examined using a Gatan cryo-transfer holder in an electron microscope (CM120; Philips Electron Optics). During transfer, examination, and imaging, the grid was maintained at ≤−180°C. Underfocused images (−1.2 to −2.0 μm) of layers of frozen solution spanning holes in the support film were recorded using low dose methods (Kodak SO163 film). Positive prints were made for further examination of the complexes.

Immunoisolation of the γTuSC

PEG was added to 3% (from a 30% stock in HB100) to clarified extract corresponding to 40 g of embryos. The mixture was incubated on ice for 20 min, centrifuged at 17,000 rpm for 10 min in the SS34 rotor at 4°C, and the supernatant was discarded. Pellets were resuspended by gentle Dounce homogenization in 40 ml ice-cold HB500 plus 100 μM GTP and clarified at 35,000 rpm for 30 min at 4°C in the 50.2 Ti rotor. γ-tubulin small complex (γTuSC) was immunoprecipitated by adding 1.46 mg of DrosC17 or DrosC12 antibody and incubating at 4°C for 1 h with gentle rotation. The immunoprecipitate was collected by pumping the antibody-extract mixture over a 500-μl column of protein A–agarose preequilibrated in HB500. The column was washed with 60 ml HB500 plus 100 μM GTP. Alternatively, the immunoprecipitate was collected by mixing the resin in a batch with the antibody-extract mixture at 4°C for 1 h. Afterwards, the resin was washed six times in batches with 5 ml of HB500 plus 100 μM GTP and loaded into a Bio-spin column. 500 μl of EB500 was loaded onto the column, the column was sealed with parafilm, and incubated for 4 h at 4°C. The γTuSC was collected by loading an additional 550 μl of EB500 onto the column and collecting the flow through. 500 μl of this eluate was fractionated on a 4.5-ml 5–20% sucrose gradient in HB500 plus 100 μM GTP at 45,000 rpm in an SW55 rotor for 10 h at 4°C. 300-μl fractions were collected from the top. For coverslip and solution nucleation assays, 100 μl of each fraction was dialyzed against HB100 plus 100 μM GTP at 4°C for 6 h. The concentration of γ-tubulin was estimated after dialysis and, in general, was not significantly altered.

Solution Nucleation Assays to Quantitate Microtubule Nucleation

5-μl reactions of identical final buffer composition (0.5× BRB80, 0.5× EB200, 500 μM GTP) containing 4 mg/ml tubulin and varying concentrations of peptide-eluted complexes were incubated at 37°C for 4 min and fixed at room temperature for 3 min by addition of 45 μl of 1% glutaraldehyde in BRB80. 10 μl was removed to a new tube and diluted by addition of 1 ml ice-cold BRB80. MT spindowns and tubulin immunofluorescence were performed as described (http://skye.med.harvard.edu). Varying amounts of each sample were pelleted depending on the concentration of γ-tubulin in the reaction. 20 random fields were photographed with a 60× objective (1.4 NA; Nikon Corp.) using a cooled CCD camera (Princeton Instruments) and the MTs were counted. The fraction of total γ-tubulin in the peptide-eluted complexes present as the γTuRC was determined by densitometry of Coomassie-stained gels after sucrose gradient fractionation.

To compare nucleating activity, peptide-eluted γ-tubulin complexes containing 0.65 μM γ-tubulin in the γTuRC (0.87 μM total γ-tubulin) and isolated γTuSC containing 0.74 μM γ-tubulin were assayed in parallel as above. The following exceptions were made: the incubation at 37°C was for 3 min; the final buffer composition of the γTuSC was 0.5× BRB80, 0.5× HB100 plus 100 μM GTP, 500 μM GTP; after fixation, instead of sedimentation, samples were diluted with 200 μl of BRB80 + 70% glycerol and 3 μl were squashed and sealed under 18-mm square coverslips.

Coverslip Nucleation Assay

Polylysine-coated 12-mm diameter coverslips were placed on parafilm inside a humidified Petri dish kept in a 30°C water bath. The coverslips were rinsed 2× with filtered water and blocked for 5 min with 60 μl HB block. The HB block was removed by aspiration and replaced with 20 μl of the sample. After 10 min the coverslips were washed 2× with 60 μl of BRB80 + 10 mg/ml BSA + 1 mM GTP, and incubated with 20 μl 6 mg/ml tubulin (1:4 rhodamine labeled/unlabeled) in BRB80 + 1 mM GTP. After 10 min the tubulin was removed by aspiration and replaced with 60 μl 1% glutaraldehyde in BRB80 (warmed to 30°C) for 3 min, followed by 3 min postfixation with −20°C methanol. The coverslips were rehydrated, mounted, and sealed with nail polish.

Cloning and Sequencing of Dgrip84 and Dgrip91

The *Drosophila* gamma ring proteins (Dgrips) were immunoisolated as described below and internal peptide sequences were obtained for Dgrip84 and Dgrip91. The following peptides were obtained for Dgrip84: KILRTGK, KDAQQLIIGLVRK, DRSLTH, DELPEHY, DIHTHL, DLVTQMS, DAEVLTYL, DEQIPSFLA, RHREFL, DFTMQ, ERRTYTLR, DTTPVVFVRRGP, DRHRE, DEYRTSLL, DEQIPSFLAKY, DVNSAAGSVPTTLAIAST, and DLVTQMSKIMKKEENXQAQ. For Dgrip91 the peptides obtained were: KDVVTGRF, KGVYGLTN, KTVSDH, KHMEFVLS, DIMVGPHK, DFNEYY, KLSELGYY, DATKMLP(OR L)E, DRVVKFS, DVIVQRPFNGG, EMIICIKGKQMPE, DVVTGRIFPY, ELSKIV, DATQSSIGLXKQSLPNY, DDPNLQLFGTR, DQSRFYK, and DVSTGFNAIG. For Dgrip84, degenerate primers corresponding to the forward peptide KDAQQL and reverse peptide DLVTQM (underlined above) specifically amplified a band of ~700 bp. A second round of PCR was performed with a primer corresponding to the forward peptide QQLIIG and the same reverse primer. For Dgrip91, primers corresponding to the forward peptide IKGKQM and reverse peptide TGFNAI (underlined above) specifically amplified a band of ~800 bp. A second round of PCR was performed with a primer corresponding to the forward peptide GKQMPE and the same reverse primer. Both PCR products were cloned, sequenced, and used to screen a *Drosophila* cDNA library.

Antibodies

Synthetic peptides (Stanford University Medical Center) corresponding to the COOH-terminal 12 (QWSPAVEASKAG; DrosC12) or 17 amino acids (QIDYPQWSPAVEASKAG; DrosC17) of the maternal form of *Drosophila* γ-tubulin (37°C; These data are available from GenBank/EMBL/DDB3 under accession number P42271) were used to raise and affinity purify rabbit polyclonal antibodies (Field et al., 1998). Antibodies to Dgrip84 and Dgrip91 were raised in rabbits against fusions of glutathione-S-transferase with amino acids 89–199 and 29–143 of the two proteins, respectively. Specific antibodies were purified as described (Kellogg and Alberts, 1992).

Embryo Fixation and Immunofluorescence

Embryos were fixed in methanol and immunofluorescence was performed as described (Theurkauf, 1994). Embryos were double-labeled with mouse anti–γ-tubulin (Sigma Chemical Co.) and rabbit anti-p91 or anti-p84 followed by FITC anti–rabbit and Cy-5 anti–mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Three-dimensional images were obtained on a wide field microscope (DeltaVision; Applied Precision Inc.). 512 × 512 pixel optical sections were taken at 0.2-μm intervals using an Olympus 60×, 1.4 NA objective and deconvolved. Appropriate Z-sections were projected.

GTP Cross-linking Experiments

120-μl peptide-eluted complexes were isolated as above, except GTP was omitted from the column wash and elution buffers. The isolated complexes were loaded onto a 2-ml 5–40% sucrose gradient in HB100, centrifuged at 55,000 rpm in a TLS55 rotor for 4 h at 4°C, and fractionated into 17 130-μl fractions. In a 96-well plate, 30 μl of each fraction was incubated for 90 min on ice with 10 μ Ci of [α-^{32}P]GTP (400 Ci/mmol; Amersham Pharmacia Biotech Inc.), cross-linked for 5 min on ice in a cross-linker (Stratalinker UV; Stratagene) at a distance of 10 cm, and analyzed by 10% SDS-PAGE followed by autoradiography. For competition experiments, the [α-^{32}P]GTP was premixed with a 200-fold excess of cold nucleotide competitor before being added to the fraction.

Determination of γTuSC Nucleotide Content

γTuSC was prepared as described above with the following modifications: 1:1,000 LPC was used in place of 1:200 protease inhibitor stock; pellets from the PEG precipitation were resuspended in buffer containing 100 μM of either GTP or GDP; and the wash, elution buffers, as well as sucrose gradients contained 20 μM of either GTP or GDP. Control gradients loaded with EB500 were fractionated in parallel to generate control buffers. αβ-tubulin samples were prepared by diluting bovine brain tubulin into control buffers and incubating 1 h on ice before desalting. Free nucleotide was removed by rapid desalting into 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$ on a 800-μl Fast Desalting column (PC 3.2/10) mounted on a SMART™ system (Pharmacia Biotech, Inc.). 100 μl of sample was loaded per run and the column was eluted at a flow rate of 400 μl/min. One 100-μl fraction containing the protein peak was collected from each desalting run. Two separate runs were pooled, generating a total desalted sample volume of 200 μl. Under these conditions, desalting was complete in ~45 s and ≥99.9% of free nucleotide was removed. 20 μl of the desalted sample was used for protein quantitation (see below). The concentration of γ- or αβ-tubulin loaded onto the desalting column was usually between 0.5 and 1 μM; protein recoveries were ~40–50%.

To extract nucleotide from the remaining desalted sample, 90 mg of solid urea was added and the sample was vortexed and heated to 50°C for 3 min to denature the protein and release bound nucleotide. The denatured protein was removed by filtration through a 10,000 mol wt cutoff filter (model UFC3LGC00, Millipore Corp.; Rosenblatt et al., 1995). The filter was washed with 150 μl of water; eluate and wash were combined and frozen in liquid nitrogen. Nucleotide content was determined by chromatography on a 100-μl Mono Q (PC 1.6/5; Pharmacia Biotech, Inc.) column mounted on the SMART™ system. Nucleotides were eluted with a gradient of ammonium bicarbonate (0.1–1 M in 30 column vol), quantified by peak integration, and compared with standard curves generated by processing nucleotide standards of known concentration in an identical fashion. The amount of γ-tubulin and αβ-tubulin was quantified using 10% SDS-PAGE, Coomassie-staining, and densitometry of the 20-μl aliquot of sample reserved after desalting relative to a standard curve of αβ-tubulin dimer on the same gel.

Results

Drosophila Contains Two Related γ-tubulin Complexes: The γTuSC Is a Subunit of the γTuRC

Drosophila embryo extracts contain two γ-tubulin–containing complexes that can be separated by gel filtration chromatography or sucrose gradient sedimentation. In the presence of 500 mM KCl or NaCl, γ-tubulin is found exclusively in the smaller complex, indicating that the larger complex has been disrupted, and that the smaller complex is likely to be a structural subunit of the larger complex (Moritz et al., 1998). We named the large complex, *Drosophila* γTuRC (see below), and the small complex the γTuSC. To obtain size estimates for each complex, we performed gel filtration and sucrose gradient sedimentation under low salt conditions in buffers that were supplemented with magnesium and GTP (Fig. 1) to reduce aggregation that occurs in nucleotide-free buffers. Under these conditions, the γTuSC has an S value of 9.8 and a Stokes radius of 7.0 nm, while the γTuRC has an S value of 35.5 S and a 15-nm Stokes radius. Based on these values, we estimate the molecular masses of the γTuSC and γTuRC to be 280,000 and 2,200,000 D (Table I), respectively.

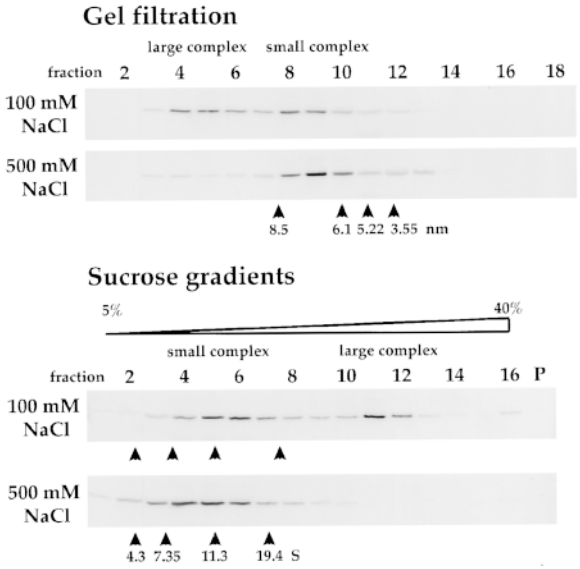

Figure 1. Hydrodynamic analysis of γ-tubulin in concentrated *Drosophila* embryo extracts. (Top) γ-tubulin immunoblots of Superose 6 gel filtration column fractions in buffer containing 100 μM GTP and 100 or 500 mM NaCl. Calibration standards for the Superose 6 column: bovine thyroglobulin (Stokes radius = 8.5 nm), horse spleen ferritin (6.1 nm), bovine liver catalase (5.22 nm), and bovine serum albumin (3.55 nm), as indicated with arrowheads. (Bottom) γ-tubulin immunoblots of 5–40% sucrose gradient fractions in buffer containing 100 μM GTP and 100 or 500 mM NaCl. Gradients were sedimented at 50,000 rpm for 4 h in an SW55 rotor at 4°C and fractionated from the top; gradient pellets are also shown (P). The peak locations of standards run on parallel gradients are indicated with arrowheads. Sucrose gradient standards: bovine serum albumin (4.3 S), rabbit muscle aldolase (7.35 S), bovine liver catalase (11.3 S), and porcine thyroglobulin (19.4S).

Purified Drosophila γ-tubulin Complexes Nucleate Microtubules In Vitro

To purify *Drosophila* γ-tubulin complexes, we used an immunoaffinity strategy based on antibodies raised against a COOH-terminal peptide (Zheng et al., 1995) of *Drosophila* γ-tubulin. Immunoprecipitated protein complexes were eluted from the antibody with buffers containing

Table I. Properties of Drosophila γ-tubulin–containing Complexes

	S value	Stokes radius	Estimated molecular mass
		nm	*D*
In extract (100 mM NaCl)			
γTuSC	12.8	7.6	390,000
γTuRC	35.5	15.0	2,200,000
In extract (500 mM NaCl)			
γTuSC	9.8	7.0	280,000
Purified (100 mM NaCl)			
γTuSC	9.7	7.3	280,000
γTuRC	31.6	n/a	
Purified (500 mM NaCl)			
γTuSC	9.3	7.3	270,000

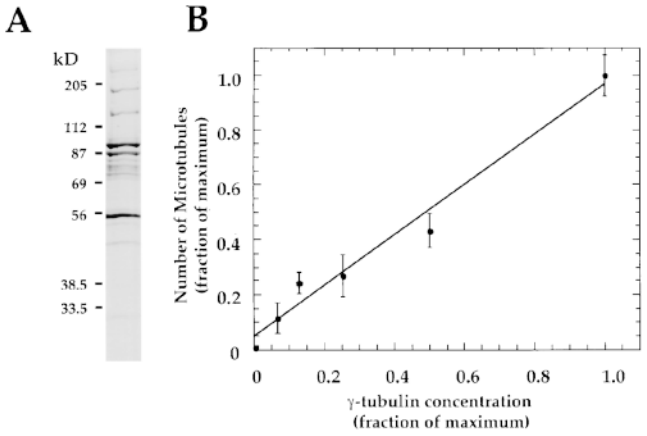

Figure 2. Purified *Drosophila* γ-tubulin complexes nucleate MTs in vitro. (A) Protein profile of peptide-eluted immunoisolated *Drosophila* γ-tubulin complexes after separation by 10% SDS-PAGE and Coomassie staining. (B) Nucleating activity is proportional to the concentration of γ-tubulin complexes in the reaction. The results shown here are the average of three independent experiments performed using the same preparation of peptide eluted complexes. At the highest concentrations tested, ~370 nM or ~0.02 mg/ml γ-tubulin, there were 94 times more MTs than in control reactions without γ-tubulin complexes. We estimate that the maximal concentration of MTs was ~0.30 nM. Error bars represent the SEM.

competing peptide (Fig. 2 A). The peptide-eluted mixture of γTuRC and γTuSC nucleates MTs in solution (Fig. 2 B). The number of MTs nucleated is directly proportional to the concentration of γ-tubulin complexes (Fig. 2 B). At the highest concentrations tested (370 nM or ~0.02 mg/ml γ-tubulin), γ-tubulin complex–containing reactions nucleated ~100-fold more MTs than control reactions. The relative proportions of γTuRC and γTuSC vary between preps. For the experiments shown in Fig. 2, 67% of the γ-tubulin was present as γTuRC, 22% was present as γTuSC, and 11% was in complexes intermediate in size.

The Protein Profile of the Drosophila γTuRC Is Similar to That of the Xenopus γTuRC

To determine the protein compositions of the γTuSC and γTuRC, we fractionated the peptide-eluted complexes on a 5–40% sucrose gradient (Fig. 3 A). For clarity, γTuSC and γTuRC are shown side by side in Fig. 3 B. The protein profile of the *Drosophila* γTuRC is reminiscent of the *Xenopus* γTuRC (Fig. 3 B). Therefore, by analogy to the Xgrips (Martin et al., 1998), we name *Drosophila* γTuRC proteins Dgrips and designate them by their apparent molecular weights. Like the *Xenopus* γTuRC, the *Drosophila* γTuRC is composed of two high molecular mass proteins (Dgrip163 and Dgrip128), two prominent proteins near 100 kD (Dgrip91 and Dgrip84), and a group of three or four proteins with molecular masses near 75 kD (Dgrip75s). The protein below γ-tubulin (between the 56- and 38.5-kD markers) has been identified as actin. It is not clear whether actin is a specific component of γTuRC, or if it fortuitously copurifies. Depending on the purification protocol, varying amounts of α- and β-tubulin copurify with *Xenopus* γTuRC (Zheng et al., 1995; Y. Zheng, unpublished results). In contrast, we have been unable to detect α- or β-tubulin copurifying with *Drosophila* γTuRC. Consistent with the idea that γTuSC is a structural subunit of γTuRC, γTuSC is composed of the three most prominent proteins in γTuRC: γ-tubulin, Dgrip84, and Dgrip91 (Fig. 3 B).

The Drosophila γTuRC Nucleates MTs In Vitro

To determine which of the *Drosophila* γ-tubulin complexes were able to nucleate MTs, we separated them by sucrose gradient sedimentation and tested them using a coverslip nucleation assay (outlined in Fig. 3 C). In this assay, the sample to be tested is allowed to bind to a preblocked coverslip, unbound protein is washed away, and the coverslip is incubated with purified bovine brain tubulin containing a small amount of rhodamine-labeled tubulin. Unincorporated tubulin and spontaneously nucleated MTs are removed by aspiration, whereas MTs nucleated and tethered to the coverslip by the γ-tubulin complexes remain and are fixed and viewed by fluorescence microscopy. Although this assay is not quantitative, it has two advantages over the conventional solution nucleation assay: (a) the background of spontaneously nucleated MTs is removed by aspiration, allowing detection of very low levels of γ-tubulin dependent nucleation; (b) buffer components are washed away before exposure to tubulin. The latter is useful when directly assaying sucrose gradient fractions because of the strong interfering effects of varying sucrose concentrations on tubulin nucleation and elongation.

The results of a coverslip assay on the sucrose gradient fractions in Fig. 3 A are shown in Fig. 3 D. The top two rows are equivalent exposures for fractions 3–14. The bottom row shows longer exposures (either 40× or 5× longer) for the indicated fractions. We observe a clear peak of activity corresponding to the fractions that contain γTuRC (fractions 10–14). Under these conditions, no activity is seen in the fractions containing γTuSC (fractions 4–6). Similar to the peptide-eluted complexes, gel filtration or sucrose gradient–fractionated *Drosophila* embryo extracts tested in this assay show only one peak of activity, corresponding to the peak of γTuRC (data not shown). To distinguish between nucleation and capture of spontaneously nucleated MTs, we performed the coverslip nucleation as-

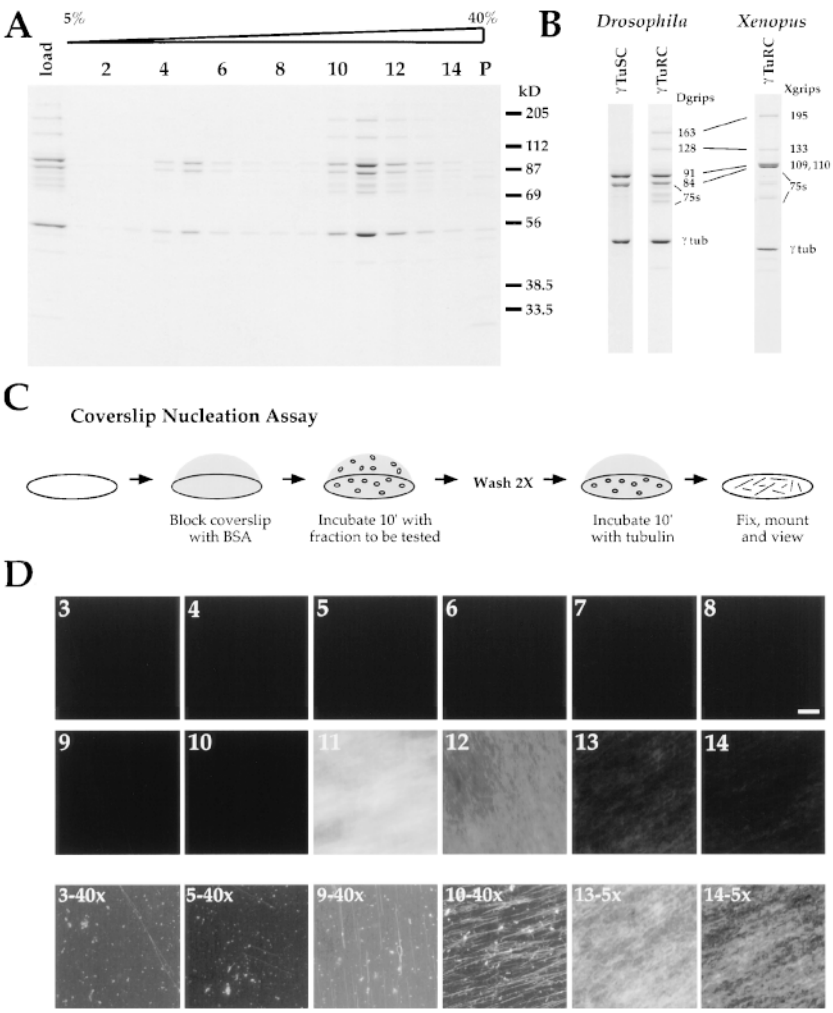

Figure 3. Characterization of γ-tubulin–containing complexes isolated from *Drosophila* embryo extracts. γ-tubulin–containing complexes were immunoisolated and fractionated by sucrose gradient sedimentation in 100 mM NaCl. (A) 75 μl of each sucrose gradient fraction was TCA precipitated and analyzed by 10% SDS-PAGE and Coomassie staining. The sucrose gradient load is also shown. Peak fractions for standards run on a parallel gradient were: BSA (4.3 S), fraction 2.7; aldolase (7.35 S), fraction 4.0; catalase (11.3 S), fraction 5.6; and bovine thyroglobulin (19.4 S), fraction 8.0. (B) Protein profiles of γTuRC and γTuSC. γTuSC consists of the three most prominent bands in γTuRC. The profile of *Drosophila* γTuRC resembles that of *Xenopus* γTuRC (Zheng et al., 1995). (C) Schematic of the coverslip nucleation assay. The coverslip is washed and blocked with a BSA-containing buffer, incubated with the sample to be tested, rinsed to remove unbound protein, incubated with a mixture of unlabeled and rhodamine-labeled tubulin, fixed, and viewed using fluorescence microscopy. (D) Analysis of sucrose gradient fractions in A using the coverslip assay. The top two rows are equivalent exposures for fractions 3–14. The bottom row shows longer exposures (either 40× or 5× longer, as indicated) for some fractions. Bar, 10 μm.

say with peptide-eluted material and observed unfixed samples in real time using video enhanced DIC microscopy (data not shown). MTs initiated at the coverslip surface and elongated, while maintaining a fixed orientation with one end anchored to the coverslip. Capture of a MT from solution by the surface was never observed. Cumulatively, these results indicate that the *Drosophila* γTuRC has MT nucleating activity.

Cryo-electron Microscopy of the γTuRC Reveals a Modular Structure

Negative stain electron microscopy of the peptide-eluted complexes (Fig. 4 A), and of the γTuRC after sucrose gradient sedimentation (Fig. 4 B) reveals an open ring structure with a diameter of ~25 nm. In side-by-side pictures of comparable preparations, the structure of the *Drosophila* γTuRC is indistinguishable from that of the *Xenopus* γTuRC (Wiese, C., and Y. Zheng, unpublished observations). To get a more detailed view of the γTuRC, we examined the structure of the purified *Drosophila* γTuRC by cryo-electron microscopy. A gallery of cryo-EM images reveals a modular structure (Fig. 4 C). The γTuRC appears to have ~13 structural repeats arranged in a radial symmetric pattern with a diameter of 25 nm. Some internal structures are also visible. A more detailed view will require single particle reconstructions.

γTuRC Is a More Potent Microtubule Nucleator than γTuSC

The absence of nucleation activity of sucrose gradient–isolated γTuSC in the coverslip assay (Fig. 3 D) can be explained in several ways: γTuSC (a) might not have nucleating activity; (b) might not bind to the coverslip under the assay conditions; (c) might become inactivated upon binding to the coverslip, or (d) might be too dilute to exhibit activity. To distinguish between these possibilities we developed a protocol to prepare more concentrated γTuSC, taking advantage of the disruption of γTuRC into γTuSC by high salt (Fig. 1). γTuRC was disrupted by isolating γ-tubulin–containing complexes in the presence of 500 mM NaCl. The resulting γTuSC was eluted with peptide containing buffer in 500 mM NaCl. The peptide-eluted material was further fractionated on a 5–20% sucrose gradient in 500 mM NaCl (Fig. 5 A). This gradient separated γTuSC from residual larger complexes and from non-γTuSC components of γTuRC. This resulted in highly concentrated, relatively pure γTuSC (15 μl of each fraction was loaded on the gel in Fig. 5 A compared with 50 μl

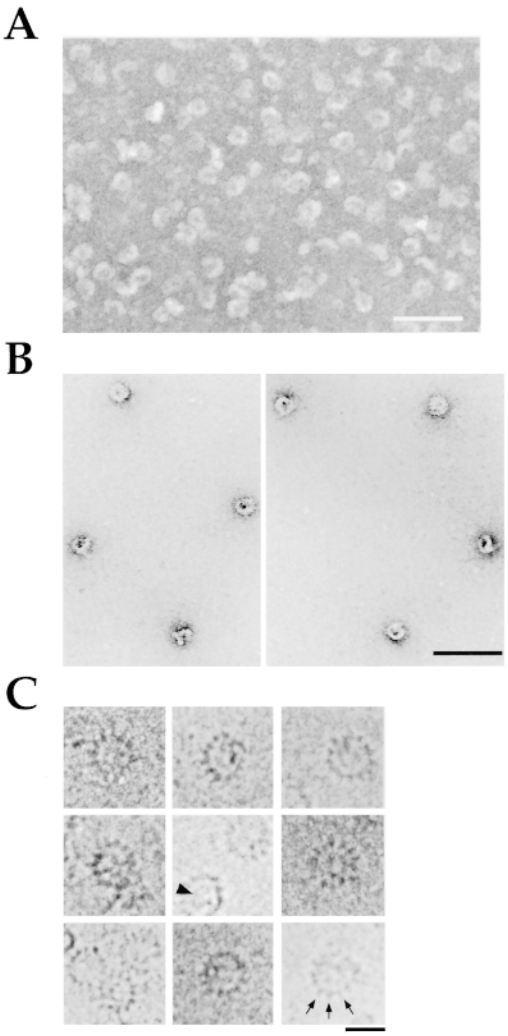

Figure 4. Structure of *Drosophila* γTuRC. (A and B) Negative stain electron microscopy of the peptide-eluted complexes (A) or of *Drosophila* γTuRC after isolation on a sucrose gradient (B). Bar, 100 nm. (C) A gallery of cryo-electron microscopy images of *Drosophila* γTuRC. Cryo-electron microscopy reveals a modular structure with ~13 structural repeats (arrows) in a radial pattern. The ring has a diameter of ~25 nm. Some internal structures are also apparent (arrowhead). Bar, 25 nm.

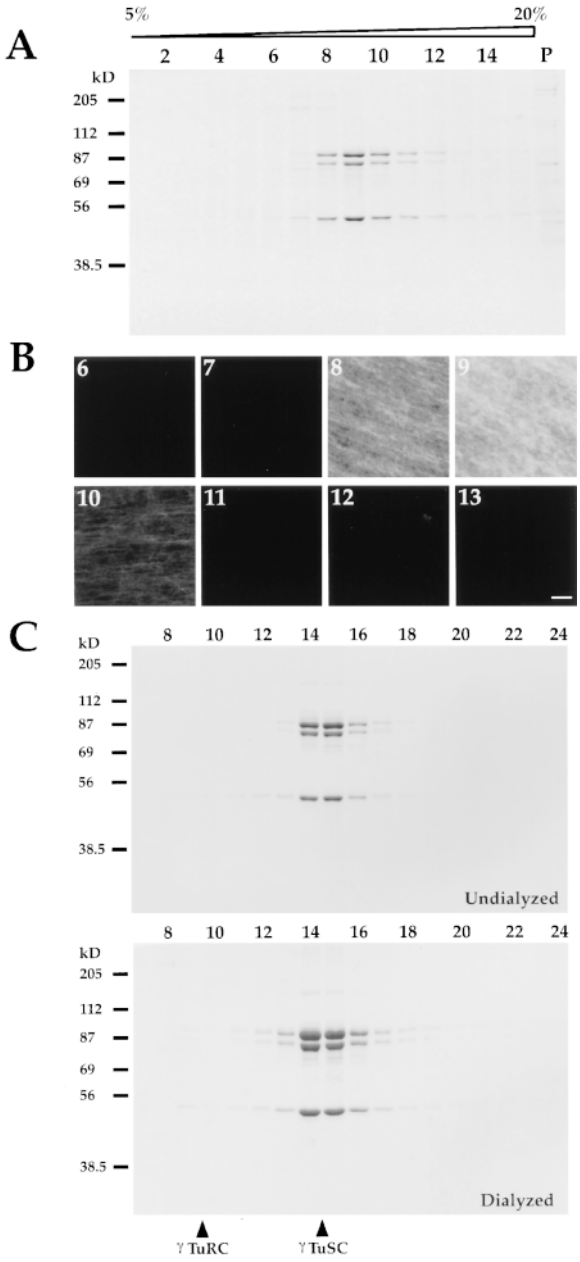

Figure 5. Characterization of the γTuSC. (A) Immunoisolated γTuSC was fractionated on a 5–20% sucrose gradient in buffer containing 500 mM NaCl. Fractions were separated by SDS-PAGE on a 10% gel and stained with Coomassie blue. A standards gradient was run in parallel. Peak fractions for standards were: BSA (4.3 S), fraction 4.9; aldolase (7.35 S), fraction 7.5; and catalase (11.3 S), fraction 10.8. (B) Fractions from the gradient in A were dialyzed against buffer containing 100 mM NaCl and tested in the coverslip assay. Bar, 10 μm. (C) γTuSC from the sucrose gradient in 500 mM NaCl was fractionated by Superose 6 gel filtration in 500 mM NaCl (top), or was first dialyzed against buffer containing 100 mM NaCl and then fractionated by Superose-6 gel filtration in buffer containing 100 mM NaCl (bottom). Standards of known Stokes radius were used to calibrate the column. The peak fractions for the gel filtration standards were: bovine thyroglobulin (8.5 nm), fraction 13.5; horse spleen ferritin (6.1 nm), fraction 15.4; bovine liver catalase (5.22 nm), fraction 16.9; aldolase (4.81 nm), fraction 17.1; ovalbumin (3.05 nm), fraction 18.0; and chymotrypsinogen (2.09 nm), fraction 20.0.

in Fig. 3 A). Inclusion of 500 mM salt in the sucrose gradient was important to prevent any reassociation of γTuSC with nonγTuSC components of γTuRC. Typically, the peak γTuSC sucrose gradient fraction contained ~700 nM γ-tubulin (as judged by densitometry of Coomassie-stained bands relative to αβ-tubulin standards). For comparison, after sucrose gradient fractionation, the peak γTuSC-containing sucrose gradient fraction in the mixed complex preparation (Fig. 3 A) contained ~70 nM γ-tubulin.

Concentrated γTuSC fractions were dialyzed to remove salt and tested for nucleating activity. In contrast to the robust activity of the peptide-eluted complexes, nucleation by isolated γTuSC in solution was weak and slightly variable between preparations. A direct comparison between the nucleating activity of the peptide-eluted complexes

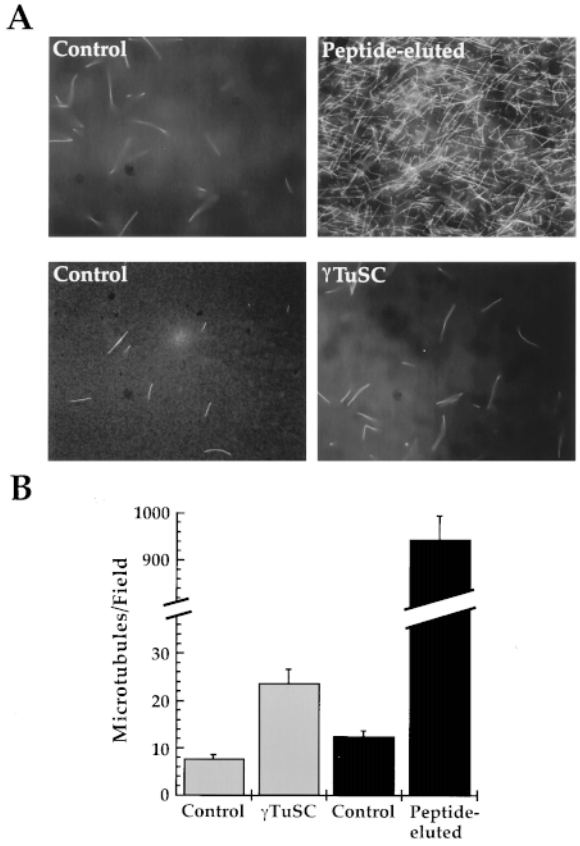

Figure 6. Comparison of the nucleating activity of peptide-eluted complexes and isolated γTuSC. (A) Representative fields from solution nucleation assays. Bar, 20 μm. (B) Quantitation of solution nucleation assays. MTs in 10 microscope fields were counted and the average number of MTs/field is plotted. Error bars represent the SEM.

(containing 0.87 μM total γ-tubulin, 0.65 μM γ-tubulin in γTuRC) and isolated γTuSC (0.74 μM γ-tubulin) in a solution nucleation assay is shown in Fig. 6. In nucleation reactions containing between 350–450 nM γ-tubulin and 4 mg/ml tubulin, the nucleating activity of the peptide-eluted complexes was typically 80–100-fold above the level of spontaneous nucleation; under these conditions the level of nucleation for isolated γTuSC was only two- to threefold above background (see Fig. 6 B). Therefore, it is likely that the nucleating activity of the peptide-eluted complexes is due to primarily the activity of γ-tubulin in γTuRC. Based on those data, we estimate that per mole of γ-tubulin γTuRC is ~25 times more active than γTuSC in promoting nucleation. Since there are ~12 γ-tubulin molecules in γTuRC and only 2 in γTuSC (see below for stoichiometry estimates), γTuRC is ~150 times more active than γTuSC per mole of complex. We also tested the nucleating activity of γTuSC in the coverslip nucleation assay. The concentrated γTuSC was also able to nucleate MTs in this assay (Fig. 5 B).

The activity of γTuSC could be explained at least two ways: (a) γTuSC might reassemble into a γTuRC-like higher-order structure, or (b) γTuSC itself might have intrinsic nucleating activity. To begin distinguishing between these possibilities, we examined dialyzed γTuSC for evidence of formation of higher-order structures using gel filtration chromatography. Undialyzed γTuSC migrated in a position typical of that for the smaller complex (Fig. 5 C, top). The dialyzed sample migrated in the same position (Fig. 5 C, bottom), suggesting that dialysis did not induce detectable assembly of γTuSC into larger structures.

Dgrip84 and Dgrip91 Are Homologous to the Spc97/Spc98 Family of Proteins

To characterize the molecular nature of γTuSC, we cloned and sequenced its non–γ-tubulin components, Dgrip84 and Dgrip91. Dgrip84 and Dgrip91 are homologous to each other and to the Spc97/98p family of proteins identified in *S. cerevisiae.* This family also includes two proteins identified in humans, hGCP2 and hGCP3 (Murphy et al., 1998). The homology between the *Drosophila* proteins and the other members of this family extends over the entire length of the proteins (data not shown). In comparisons of Dgrip84 and Dgrip91 with the corresponding human proteins, a one-to-one correspondence emerges. Dgrip84 is 32% identical (46% similar) to hGCP2 and only 21% identical (32% similar) to hGCP3; in contrast, Dgrip91 is 31% identical (45% similar) to hGCP3 and only 24% identical (37% similar) to hGCP2. These results suggest that Dgrip84 and hGCP2, and Dgrip91 and hGCP3 may be functionally homologous pairs. The sequences of the *Drosophila* proteins are available from GenBank/EMBL/DDBJ under accession numbers AF118379 (Dgrip84) and AF118380 (Dgrip91).

Stoichiometry of Proteins in γTuSC

To estimate the stoichiometry of γTuSC proteins, we performed densitometry of Coomassie-stained SDS-PAGE gels of purified γTuSC. After correcting for the predicted molecular weight of each protein, we estimated that the ratio of Dgrip91 to Dgrip84 to γ-tubulin in the γTuSC is 1:1:2. Since our estimate of the molecular mass of purified γTuSC from sucrose gradient sedimentation and gel filtration is 280,000 D (Table I), we suspect that γTuSC contains 1 molecule of Dgrip91, 1 molecule of Dgrip84, and 2 molecules of γ-tubulin. Interestingly, this corresponds to estimates of the stoichiometry of proteins in the *S. cerevisiae* 6 S γ-tubulin complex (Knop et al., 1997; Knop and Schiebel, 1997). If we assume that γTuRC contains only one molecule of each non-γTuSC component, and use our estimates for the molecular weights of γTuRC and γTuSC, then γTuRC would contain approximately six γTuSCs.

Dgrip84 and Dgrip91 Cofractionate with γ-tubulin on Sucrose Gradients and Colocalize with γ-tubulin in Embryos

If γ-tubulin in *Drosophila* embryos primarily exists associated with Dgrip84 and Dgrip91 in either γTuSC or γTuRC, we would expect these three proteins to cofractionate on sucrose gradients of embryo extract and to colocalize in embryos. To test this hypothesis, we raised and affinity-purified rabbit polyclonal antibodies that recognize Dgrip84 and Dgrip91. Each antibody recognizes a band of the expected molecular weight on Western blots of embryo extract (Fig. 7 A, left). As expected, both

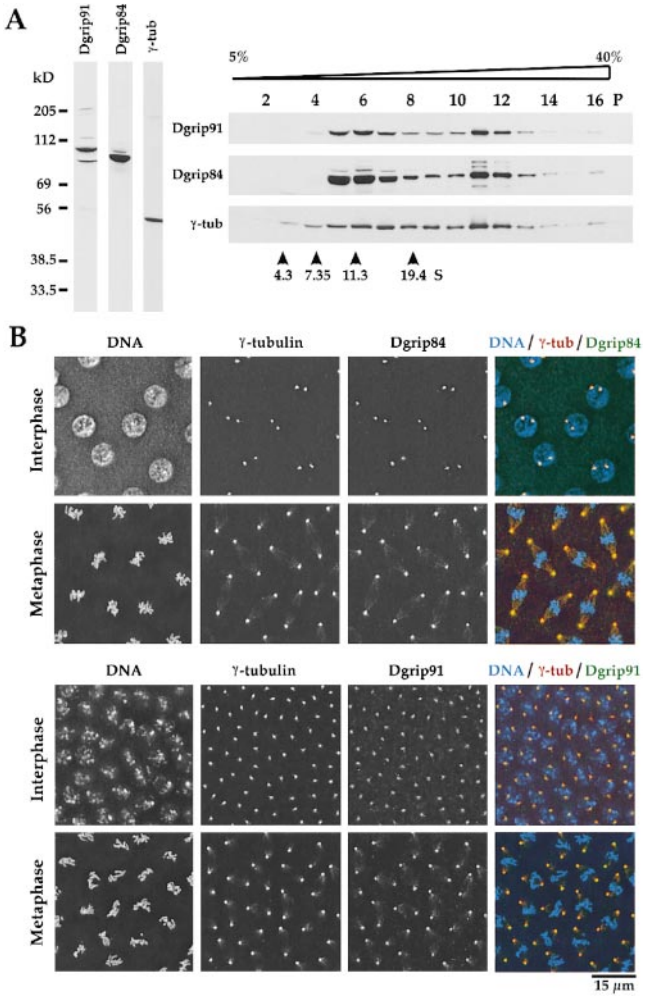

Figure 7. Dgrip91 and Dgrip84 associate and colocalize with γ-tubulin. (A) Clarified *Drosophila* embryo extract was immunoblotted for Dgrip91, Dgrip84, and γ-tubulin after separation on 10% SDS-PAGE gels (left). Each antibody recognizes a band of the expected molecular weight. Both Dgrip91 and Dgrip84 comigrate with γ-tubulin on sucrose gradients of embryo extract (right). (B) Immunofluorescence of early *Drosophila* embryos with antibodies against Dgrip91, Dgrip84, and γ-tubulin. Both Dgrip84 (top) and Dgrip91 (bottom) localize to centrosomes and to the mitotic spindle in a fashion that is indistinguishable from the localization of γ-tubulin. Examples of embryos in interphase and mitosis are shown for each antibody. Bar, 15 μm.

Dgrip84 and Dgrip91 comigrate with γ-tubulin in γTuSC and γTuRC when embryo extract is fractionated on sucrose gradients (Fig. 7 A, right). In addition, the localizations of Dgrip84 and Dgrip91 in *Drosophila* embryos are indistinguishable from that of γ-tubulin. Each antibody recognizes the centrosome throughout the cell cycle and shows some spindle staining during mitosis with enrichment at the spindle poles (Fig. 7 B), regardless of its cognate antigen. We propose that *Drosophila* γ-tubulin is stably associated with Dgrip91/84. Interestingly, we found no evidence for a non–γ-tubulin associated pool of either Dgrip84 or Dgrip91.

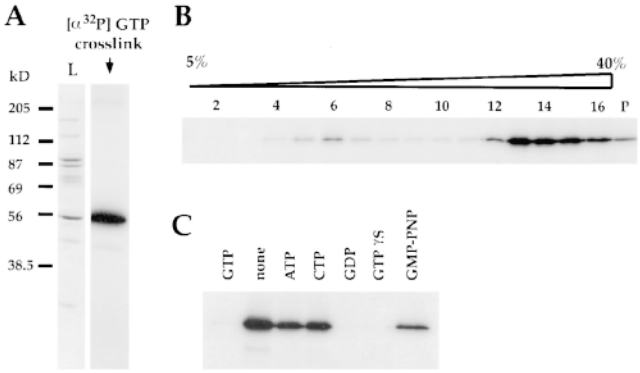

Figure 8. Photo cross-linking of radiolabeled GTP to γ-tubulin in γTuSC and γTuRC. (A) GTP cross-linking of peptide-eluted complexes. γ-tubulin is the only protein that cross-links to GTP. (B) GTP cross-linking after sucrose gradient fractionation of the material in A, demonstrating that γ-tubulin in both γTuSC and γTuRC cross-links to GTP. (C) Competition of the GTP cross-link of the material in A with 200-fold excess of the indicated unlabeled nucleotides.

γ-tubulin in γTuRC and γTuSC Can be Cross-linked to GTP

The homology between γ-, α-, and β-tubulins extends into domains that are involved in GTP binding by α- and β-tubulin (Burns, 1995). Thus, it is tempting to speculate that γ-tubulin can bind, and possibly hydrolyze, GTP. To determine if γ-tubulin binds guanine nucleotide, we immunoisolated γ-tubulin–containing complexes in the absence of GTP. The isolated complexes, either before or after sucrose gradient sedimentation, were incubated with [α-^{32}P]GTP and UV cross-linked. In the peptide-eluted complexes, γ-tubulin is the only protein that cross-links to GTP (Fig. 8 A). Furthermore, γ-tubulin in both the γTuRC and γTuSC cross-links to GTP (Fig. 8 B). Competition experiments showed that the cross-link can be competed by addition of excess cold GTP, GDP, and GTPγS but not GMP-PNP, ATP, or CTP (Fig. 8 C).

γ-tubulin in γTuSC Preferentially Binds GDP

To characterize the nucleotide binding properties of γ-tubulin, we compared the nucleotide content of γ-tubulin in γTuSC to that of similarly treated αβ-tubulin dimer. γTuSC was isolated in buffers containing either 20 μM GDP or 20 μM GTP. To remove free nucleotide, we used a rapid (within 45 s) microscale desalting procedure. For comparison, pure αβ-tubulin dimer was diluted into a buffer identical to that containing γTuSC and desalted in parallel. Nucleotide was extracted from the desalted samples and analyzed by mono Q chromatography. To estimate the stoichiometry of bound nucleotide to protein, the amount of γ- and αβ-tubulin in each desalted sample was quantitated by densitometry of Coomassie-stained gel bands relative to αβ-tubulin standards, and the nucleotide concentration was determined by peak integration and comparison with nucleotide standards processed in an identical fashion.

Each αβ-tubulin dimer has two guanine nucleotide

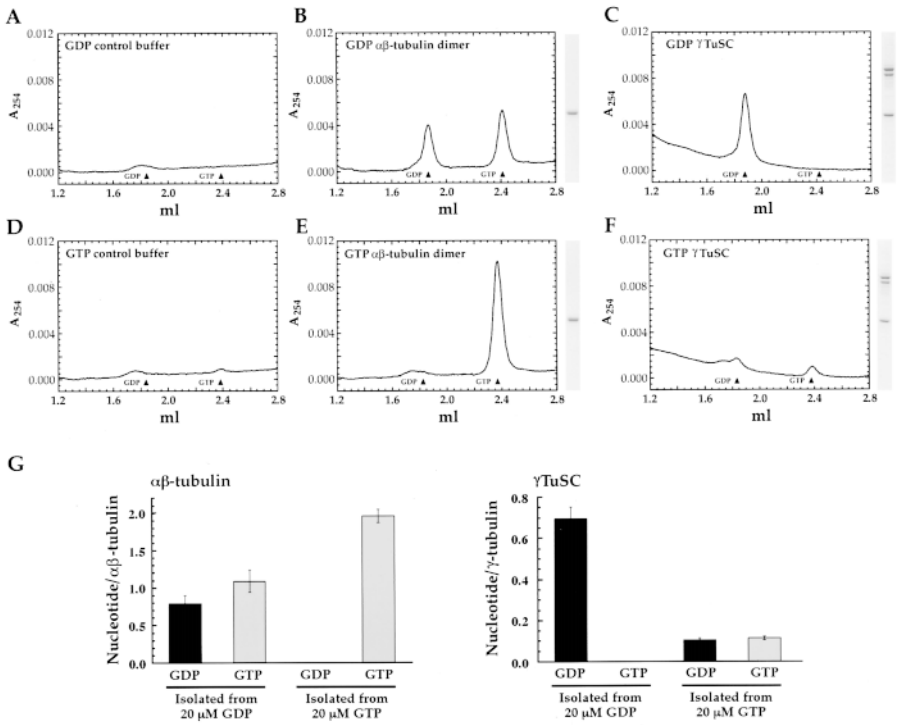

Figure 9. Analysis of nucleotide bound to γ-tubulin in γTuSC, a comparison with αβ-tubulin dimer. Desalting was used to remove free nucleotide from samples of purified γTuSC or αβ-tubulin. Bound nucleotide was released by urea treatment and analyzed on a mono Q column. Arrows indicate the elution positions of GDP and GTP. For samples containing protein (B, C, E, and F), a gel lane of the desalted sample is shown to the right of the UV trace. (A–C) Nucleotide analysis after isolation from buffer containing 20 μM GDP. (A) After desalting of control buffer no nucleotide is detected (the broad peak just to the left of the GDP arrow is a background peak). (B) Both GDP and GTP are bound to αβ-tubulin dimer (for quantitation see Table II: 20 μM GDP, experiment 3). (C) Exclusively, GDP binds to γ-tubulin in γTuSC (for quantitation see Table II: 20 μM GDP, experiment 3). (D–F) Nucleotide analysis after isolation from buffer containing 20 μM GTP. (D) Although GTP is desalted slightly less efficiently than GDP (compare with A), >99.9% of free GTP is removed from control buffer. (E) Exclusively, GTP binds αβ-tubulin dimer (for quantitation see Table II: 20 μM GTP, experiment 1). (F) Small amounts of both GDP and GTP are detected bound to γ-tubulin in the γTuSC (for quantitation see Table II: 20 μM GTP, experiment 1). (G) Summary of nucleotide analysis from three independent experiments (raw data shown in Table II). Bar graphs indicate the ratio of bound nucleotide per αβ-tubulin dimer, or γ-tubulin monomer in γTuSC after isolation from buffers containing 20 μM GDP or 20 μM GTP. Error bars represent the SEM. The ratio of GDP/GTP recovered when we desalt αβ-tubulin dimer from buffer containing GDP is very reproducible (0.733 ± 0.015, $n = 5$) suggesting that the protein concentration determined by densitometry is the least accurate parameter in this analysis. This ratio also suggests that we are recovering ~73% of the GDP bound to the β-tubulin E-site.

binding sites, one on each tubulin subunit. Exclusively GTP is bound to α-tubulin at the nonexchangeable or N-site; this nucleotide does not exchange with GTP/GDP in solution and does not undergo hydrolysis. In contrast, β-tubulin binds guanine nucleotide in an exchangeable fashion at the E-site. Both GTP and GDP bind to the E-site with GTP having a three- to fourfold higher affinity than GDP (Zeeberg and Caplow, 1979). GTP bound to the E-site does not undergo significant hydrolysis in the absence of polymerization but gets hydrolyzed soon after incorporation into the MT lattice, resulting in GDP that is locked in the lattice and can only exchange after depolymerization (reviewed in Desai and Mitchison, 1997). These properties predict that if αβ-tubulin dimer is isolated from buffers containing GDP, then there will be 1 mol GTP (N-site) and 1 mol GDP (E-site) per mole of αβ-tubulin dimer. In contrast, if αβ-tubulin dimer is isolated from buffers containing GTP, under conditions where there is no polymerization, then there will be 2 mol GTP (1 N-site GTP and 1 E-site GTP) per mole of αβ-tubulin dimer. Consistent with these predictions, we recovered 1.1 mol of GTP and 0.8 mol GDP per mole of αβ-tubulin dimer isolated from GDP buffer (Fig. 9, B and G, and Table II); in contrast, we recovered exclusively 2.0 mol of GTP per mole of αβ-tubulin dimer isolated from GTP buffer (Fig. 9, E and G, and Table II). These results establish the validity of our assay for comparing the nucleotide-binding properties of γTuSC to those of αβ-tubulin dimer.

When γTuSC was similarly analyzed, the nucleotide recovered from γTuSC incubated in GDP buffers was exclusively GDP (Fig. 9 C). Approximately 0.7 mol GDP was recovered per mole of γ-tubulin (Fig. 9 G and Table II). The exclusive presence of GDP could be explained at least three ways: (a) the guanine nucleotide binding site on γ-tubulin subunits of γTuSC is freely exchangeable; (b) GDP is locked nonexchangeably into γ-tubulin subunits of γTuSC, analogous to GTP bound at the N-site in α-tubulin; or (c) GDP is locked nonexchangeably into γTuSC as the product of earlier GTP hydrolysis, much like β-tubulin bound GDP within the body of a polymerizing MT.

To distinguish between these possibilities, we isolated γTuSC from GTP-containing buffer. Surprisingly, we recovered a greatly reduced amount of nucleotide (Fig. 9 F). Only 0.2 mol guanine nucleotide was recovered per mole of γ-tubulin, indicating that ~80% of the γ-tubulin was empty at its nucleotide binding site (Fig. 9 G and Table II). To ascertain that the GTP in the buffer had not been degraded, we removed an aliquot before desalting and analyzed its nucleotide content. This sample contained the expected amount of GTP and a trace amount of GDP (~3% of total guanine nucleotide). This amount of GDP was also recovered from a similarly processed control buffer, indi-

Table II. Analysis of Nucleotide Bound to γ-tubulin in γTuSC

Nucleotide in isolation buffer	γ-tubulin	γ-tubulin bound GDP	γ-tubulin bound GTP	αβ-tubulin	αβ-tubulin bound GDP	αβ-tubulin bound GTP
	pmol	*pmol*	*pmol*	*pmol*	*pmol*	*pmol*
20 μM GDP						
1	60	47	0	79	82	109
2	79	56	0	45	35	45
3	64	39	0	43	27	39
20 μM GTP						
1	46	4.7	4.7	44	0	81
2	43	3.7	5.7	88	0	186
3	30	3.9	3.7	27	0	55
20 μM GTP then add 20 μM GDP						
1	36	16	0.7	25	4.1	32

cating that it did not arise from hydrolysis by γTuSC or a contaminating GTPase (not shown). The low recovery of guanine nucleotide bound to γTuSC isolated from GTP buffer indicates that GDP is bound exchangeably to γ-tubulin in γTuSC. This result also argues against the theory that the GDP bound to γ-tubulin in γTuSC, isolated from GDP buffer, is being generated by earlier GTP hydrolysis. The recovery of nearly 1 mol GDP per mole of γ-tubulin from GDP buffer and the nearly equivalent amounts of GTP and GDP in the 0.2 mol nucleotide recovered per mole of γ-tubulin from GTP buffer, despite a GTP/GDP ratio ≥30 before desalting, strongly suggest that γ-tubulin in γTuSC has an exchangeable guanine nucleotide binding site that has a much higher affinity for GDP than GTP. To test this further, we isolated γTuSC in buffer containing 20 μM GTP and, 1 h before desalting, added 20 μM GDP. Consistent with our interpretation, we recovered 0.44 mol GDP and 0.02 mol GTP per γ-tubulin monomer (Table II).

Discussion

γ-tubulin Complexes in Eukaryotes

The γ-tubulin in *Drosophila* embryo extracts exists in two related complexes of ~280,000 (γTuSC) and 2,200,000 D (γTuRC). In contrast, when extracts of *Xenopus* eggs (Stearns and Kirschner, 1994; Zheng et al., 1995), *Xenopus* XTC cells (Stearns and Kirschner, 1994), human 293 cells (Stearns and Kirschner, 1994), or mouse fibroblasts (Murphy et al., 1998) were fractionated, only one complex was observed sedimenting at ~32 S (Murphy et al., 1998). The significance of this finding is not clear, but might reflect a difference between γ-tubulin–containing complexes isolated from different organisms and cell types. Interestingly, γ-tubulin in the polarized human intestinal epithelial cell line Caco-2 was present in both 10 S and 29 S complexes (Meads and Schroer, 1995). In Caco-2 cells, γ-tubulin localizes both to centrosomes and to a diffuse layer beneath the apical membrane (Meads and Schroer, 1995). However, it is not yet clear whether the presence of an apical layer of γ-tubulin correlates with the existence of a smaller 10 S complex. Further experiments will be required to determine if the differences in γ-tubulin–containing complexes present in cellular extracts are caused by different extraction conditions, or if they reflect real diversity between systems and cell types in the nature and function of γ-tubulin complexes in vivo.

The protein profile of the purified *Drosophila* γTuRC is very similar to that of the previously purified *Xenopus* γTuRC (Zheng et al., 1995). Indeed, the protein profiles of γ-tubulin complexes immunoprecipitated from a number of sources, including mouse cells after metabolic labeling (Murphy et al., 1998) and sheep brain tubulin preparations (Detraves et al., 1997), bear a strong resemblance. In addition to molecular similarities, the *Drosophila* γTuRC also resembles the *Xenopus* γTuRC in its structure (both complexes appear as open rings when visualized by negative stain electron microscopy).

γTuSC: a Conserved Subcomplex of the γTuRC

Drosophila γTuRC can be converted to γTuSC by treatment with high salt, suggesting that γTuSC is a structural subunit of γTuRC. A similar dissociation by high salt has been reported for human and *Xenopus* large γ-tubulin complexes (Stearns and Kirschner, 1994; Meads and Schroer, 1995; Zheng et al., 1995). The hypothesis that γTuSC is a subunit of γTuRC is supported by our finding that purified γTuSC is composed of the three proteins most prominent in γTuRC: γ-tubulin, Dgrip84, and Dgrip91. Dgrip84 and Dgrip91 are members of the Spc97/98p family of proteins. This family includes hGCP2 and hGCP3/HsSpc98p from humans (Murphy et al., 1998; Tassin et al., 1998) and Xgrip109 from *Xenopus* (Martin et al., 1998). Homologous ESTs have also been identified in mouse, zebrafish, and rice (Martin et al., 1998; Tassin et al., 1998). Genetic evidence in *S. cerevisiae* suggests that this family of proteins interacts directly with γ-tubulin (Geissler et al., 1996; Knop et al., 1997). Based on molecular weight estimates and densitometry of Coomassie stained gels, we estimate that *Drosophila* γTuSC is a heterotetrameric complex containing one Dgrip84, one Dgrip91, and two molecules of γ-tubulin. This stoichiometry is identical to the proposed composition of the *S. cerevisiae* 6 S complex (Sc γTuSC). Immunoprecipitation experiments with tagged

proteins indicate that Sc γTuSC contains one Spc97p, one Spc98p, and two or more molecules of γ-tubulin (Knop et al., 1997; Knop and Schiebel, 1997). Together, these results support the hypothesis that the organization of γ-tubulin into γTuSC is likely to be conserved among all organisms where γ-tubulin is found. One question of fundamental importance that needs to be addressed in the future is how the two γ-tubulin molecules within γTuSC are arranged. Are they arranged in a head to tail dimer as would be suggested by the model proposing that γTuRC is a protofilament of γ-tubulin (Erickson and Stoffler, 1996), or are they arranged in a side-by-side configuration (Zheng et al., 1995)?

In metazoa, γTuSC is further assembled into γTuRC. Based on our hydrodynamic analysis of *Drosophila* complexes, we estimate that γTuRC contains approximately four to six γTuSCs. An exact determination awaits a more accurate appraisal of the molecular weight of γTuRC, currently being attempted using scanning transmission electron microscopy. It will also be interesting to identify the structural correlate of γTuSC within γTuRC. When viewed by cryo-electron microscopy, γTuRC has a modular structure with ~13 structural repeats organized in a radial symmetric pattern. Based on our current estimates, we speculate that one γTuSC might correspond to two of the radial symmetric structural repeats visible by cryo-electron microscopy.

Nucleation Activity of γTuSC and γTuRC

An important issue with respect to the in vivo roles of γTuSC and γTuRC is their relative MT nucleating activity. The fact that *S. cerevisiae* does not appear to contain a γTuRC-like complex raises the question of whether Sc γTuSC has nucleating activity or whether it must assemble into a larger structure at the spindle pole body to become active. Conversely, in metazoa it is possible that γTuRC is a storage form for γ-tubulin and it could be γTuSC that nucleates MTs at centrosomes (Knop and Schiebel, 1997). To begin to address this question, we compared the nucleating activity of peptide-eluted complexes (in which ~75% of γ-tubulin is γTuRC) to isolated γTuSC at similar concentrations of γ-tubulin. Using both solution and coverslip nucleation assays, we found that both preparations had nucleating activity. However, whereas the solution nucleating activity of the peptide-eluted complexes was robust, typically 80–100-fold above the level of spontaneous nucleation, under similar conditions the level of nucleation for isolated γTuSC was only two- to threefold above background. Thus, per mole of γ-tubulin γTuRC is ~25 times more active than γTuSC in promoting nucleation. Combining these data with our stoichiometry measurements, we estimate that per mole of complex γTuRC is ~150 times more active than γTuSC, suggesting that organization of γTuSC into γTuRC facilitates MT nucleation activity.

We emphasize that the nature of the nucleating activity of γTuSC is still unclear. γTuSC may have intrinsic nucleating activity or it may need to assemble into larger γTuRC-like complexes in order to nucleate MTs. In *Xenopus* extracts, high-salt dissociated γTuRC components can be reassembled by desalting. This reassembly is blocked by depleting Xgrip109, suggesting that intact γTuSC is required for assembly of γ-tubulin into a γTuRC-like structure (Martin et al., 1998). Here we separate γTuSC from the remaining components of γTuRC and do not find any evidence for assembly of larger structures after desalting. This occurrence suggests that γTuSC is required but not sufficient for assembly of a γTuRC-like structure. We cannot exclude the possibility that γTuSC, like αβ-tubulin dimer, assembles into larger complexes when the temperature is raised during nucleation assays. Because the level of activity of γTuSC is very low compared to that of γTuRC, we also cannot rule out the possibility that the nucleating activity of γTuSC depends on trace levels of other γTuRC components that contaminate our γTuSC preps. Expression and purification of the γTuSC will be necessary to further characterize γTuSC activity.

Nucleotide Binding Properties of γ-tubulin

Cross-linking experiments showed that γ-tubulin in both γTuSC and γTuRC can bind guanine nucleotide. To investigate how γ-tubulin compares to other members of the tubulin family in its nucleotide binding properties, we compared the nucleotide bound to γ-tubulin in γTuSC to that bound to αβ-tubulin dimer after desalting from buffers containing GDP or GTP. We found, like β-tubulin (Weisenberg et al., 1976), γ-tubulin in γTuSC binds guanine nucleotide exchangeably. However, in contrast to γ-tubulin, which has about a threefold higher affinity for GTP than GDP (Zeeberg and Caplow, 1979), γ-tubulin in γTuSC strongly prefers binding GDP to GTP. Our results suggest that the affinity of γ-tubulin for GTP is much lower than that of β-tubulin, based on nucleotide recovery after desalting under similar conditions (Zeeberg and Caplow, 1979). Determination of the absolute affinities of γ-tubulin for GDP and GTP will be important to know whether the affinities of γ- and β-tubulin for GDP are similar. If they are, this will suggest that the strong preference of γ-tubulin for GDP is primarily because of a reduction in its affinity for GTP relative to β-tubulin. These experiments will require a reliable supply of γTuSC, currently limited by antibody availability for immunoisolation and by lack of an expressed source. A structural comparison of the nucleotide binding pocket of γ-tubulin to those of α- and β-tubulin should also be revealing. Development of procedures to prepare more concentrated and highly purified γTuRC should allow a comparison of nucleotide binding by γ-tubulin in γTuRC to that in the γTuSC. If the nucleotide binding properties of γ-tubulin in γTuRC are similar to those of γ-tubulin in γTuSC, it will suggest that GTP hydrolysis by γ-tubulin may not be important for its function in vivo.

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