Characterization and Reconstitution of *Drosophila* γ-Tubulin Ring Complex Subunits

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Abstract. The γ-tubulin ring complex (γTuRC) is important for microtubule nucleation from the centrosome. In addition to γ-tubulin, the *Drosophila* γTuRC contains at least six subunits, three of which [*Drosophila* gamma ring proteins (Dgrips) 75/d75p, 84, and 91] have been characterized previously. Dgrips84 and 91 are present in both the small γ-tubulin complex (γTuSC) and the γTuRC, while the remaining subunits are found only in the γTuRC. To study γTuRC assembly and function, we first reconstituted γTuSC using the baculovirus expression system. Using the reconstituted γTuSC, we showed for the first time that this subcomplex of the γTuRC has microtubule binding and capping activities. Next, we characterized two new γTuRC subunits, Dgrips128 and 163, and showed that they are centrosomal proteins. Sequence comparisons among all known γTuRC subunits revealed two novel sequence motifs, which we named grip motifs 1 and 2. We found that Dgrips128 and 163 can each interact with γTuSC. However, this interaction is insufficient for γTuRC assembly.

Key words: centrosome • microtubule • γ-tubulin • grip • *Drosophila*

Introduction

Microtubules (MTs)[1] are polymers assembled from α- and β-tubulin heterodimers that are essential for intracellular transport during interphase and chromosome segregation during mitosis. MTs are nucleated from microtubule organizing centers (MTOC). In animal cells, the primary MTOC is called the centrosome, which consists of two centrioles embedded in a matrix of pericentriolar material that participates in microtubule nucleation. γ-Tubulin is a highly conserved member of the tubulin superfamily (Wiese and Zheng, 1999) that localizes to MTOCs. Genetic and biochemical studies in a number of systems have shown that γ-tubulin is important for MT nucleation (Wiese and Zheng, 1999).

In higher eukaryotes, the majority of the cytoplasmic γ-tubulin appears to exist as a protein complex of ~32 S that has a distinct ring structure when viewed by electron microscopy (Zheng et al., 1995; Oegema et al., 1999). On the basis of the ring structure, this 32 S γ-tubulin complex is known as the γ-tubulin ring complex (γTuRC). The *Drosophila* and *Xenopus* γTuRCs consist of γ-tubulin and at least six other subunits referred to as gamma ring proteins, or grips (Martin et al., 1998; Oegema et al., 1999). Three characterized *Drosophila* gamma ring proteins, (Dgrips) 75/d75p (Fava et al., 1999), 84, and 91 share some sequence homology with the *Xenopus* grip, Xgrip109. Furthermore, these three *Drosophila* grips, as well as their homologues in other organisms, are centrosomal proteins (Murphy et al., 1998; Tassin et al., 1998).

The finding that the γTuRC could nucleate MTs in vitro provided insight into the mechanism of MT nucleation and led to the hypothesis that γTuRC is the major MT nucleator at the centrosome (Zheng et al., 1995; Oegema et al., 1999). In support of this hypothesis, hundreds of γTuRC-like rings were found at the pericentriolar material of *Drosophila* (Moritz et al., 1995a,b) and *Spisula* centrosomes (Schnackenberg et al., 1998). The existence of these rings correlated with the ability of the centrosomes to nucleate MTs (Schnackenberg et al., 1998). Furthermore, γTuRC is required for MT nucleation from centrosomes assembled in vitro (Felix et al., 1994; Martin et al., 1998; Moritz et al., 1998; Schnackenberg et al., 1998). In addition to nucleating microtubules, γTuRC caps the minus ends of MT in vitro (Zheng et al., 1995; Wiese and Zheng, 2000) and is found at the minus ends of MTs nucleated in its presence (Wiese and Zheng, 2000). Therefore, understanding the composition, assembly, and function of the γTuRC is important for the study of MT nucleation at the molecular level.

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[1]*Abbreviations used in this paper:* β-ME, β-mercaptoethanol; Dgrip, *Drosophila* gamma ring protein; EGS, ethylene glycol bis-succinimidyl succinate; γTuRC, γ-tubulin ring complex; γTuSC, γ-tubulin small complex; MT, microtubule.

Analysis of the *Drosophila* γ-tubulin containing complexes has shed some light on the structural organization of the γTuRC. In addition to being a component of the γTuRC, some *Drosophila* γ-tubulin is found in a complex of ~10 S known as the γ-tubulin small complex (γTuSC) (Oegema et al., 1999). The γTuSC is a tetramer composed of two γ-tubulin molecules and one each of Dgrips84 and 91 (Oegema et al., 1999). Dgrips84 and 91 share sequence homology to the yeast proteins, Spc97p and Spc98p, which, along with Tub4p (γ-tubulin homologue), form the 6 S Tub4p complex that is analogous to the *Drosophila* γTuSC (Knop et al., 1997; Knop and Schiebel, 1997).

Previous studies suggested that the γTuSC is a structural subunit of the γTuRC (Oegema et al., 1999). Approximately six γTuSCs are present in one γTuRC, where each γTuSC may correspond to two subunits of the ring wall as revealed by cryo-electron microscopy images of the γTuRC (Oegema et al., 1999). Recently, a three-dimensional reconstruction of the *Drosophila* γTuRC showed that the ring wall, covered by a cap-like structure on one face of the ring, is composed of repeated hairpin-like subunits (Moritz et al., 2000). Based on these studies, a structural model for γTuRC was proposed in which each hairpin-like subunit of the ring wall corresponds to one γTuSC, and the cap-like structure is composed of the remaining grips, Dgrips75s, 128, and 163 (Moritz et al., 2000).

The *Drosophila* γTuSC is a stable complex that remains intact in the presence of salt concentrations up to 700 mM (Oegema et al., 1999). In addition, the purified γTuSC does not appear to self assemble into a γTuRC size complex in vitro (Oegema et al., 1999), suggesting that one or more of the remaining Dgrips (Dgrips75s, 128, and 163) is required for the assembly of multiple γTuSCs into γTuRC. Here we report the identification of two subunits of the *Drosophila* γTuRC, Dgrips128 and 163. We show that γ-tubulin and Dgrips84, 91, 128, and 163 can be expressed as soluble proteins alone or in combination in the baculovirus expression system. Using this system, we have reconstituted the γTuSC and further characterized its function in vitro.

Materials and Methods

Buffers and Reagents

HB (mM): 50 Hepes, pH 8, 1 $MgCl_2$, 1 EGTA, 1 β-mercaptoethanol (β-ME), 0.1 GTP, and protease inhibitor stock at 1:200 final dilution. HB100, HB150, HB250, HB500, and HB1M (mM): HB plus 100, 150, 250, 500, or 1,000 NaCl, respectively. BRB80 (mM): 80 K-Pipes, pH 6.8, 1

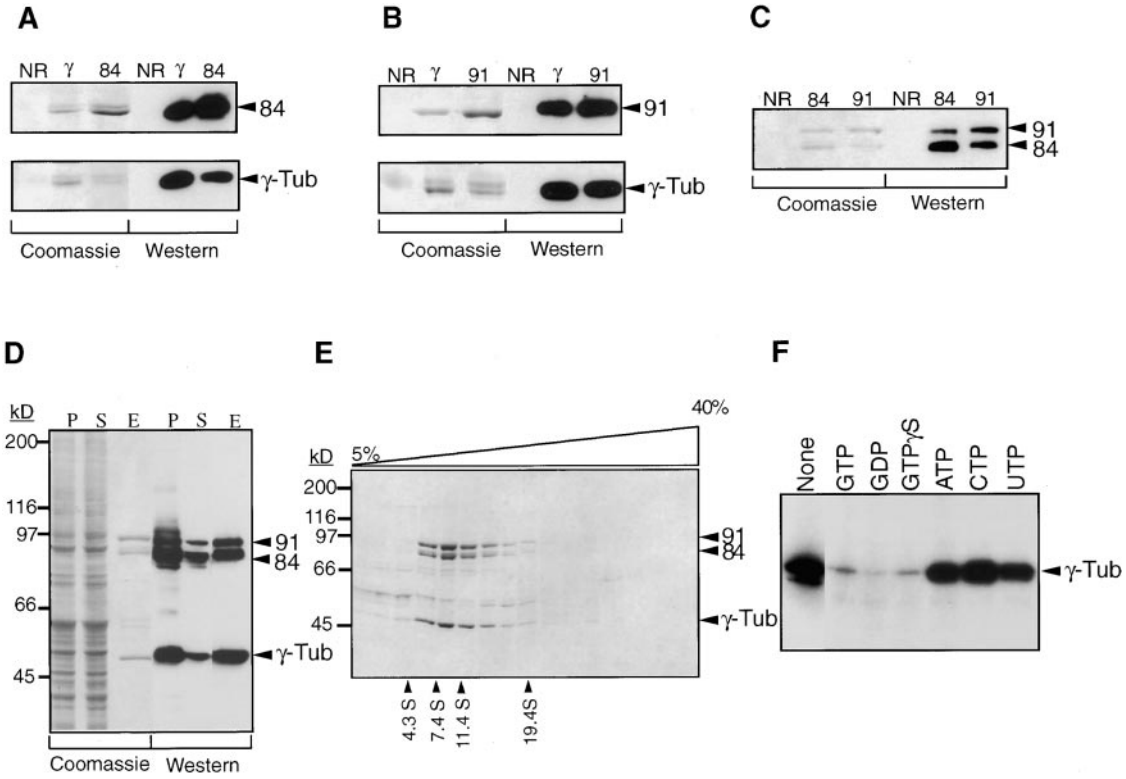

Figure 1. The interactions of the γTuSC components, its reconstitution, and purification. (A–C) *Drosophila* γ-tubulin was coexpressed with Dgrips84 (A) or 91 (B). Dgrips84 and 91 were coexpressed in the absence of γ-tubulin (C), immunoprecipitated with control antibodies (NR) or specific antibodies against γ-tubulin (γ) and Dgrips84 (84) and 91 (91). The immunoprecipitates were analyzed on SDS gels by Western blotting and Coomassie blue staining. (D) Reconstituted γ-complex was immunoisolated from Sf9 cells coexpressing Flag-γ-tubulin and Dgrips84 and 91 using the Flag antibody. The cell pellet (P), soluble fraction (S), and eluted γ-tubulin complex (E) were subjected to 10% SDS-PAGE and either stained by Coomassie blue or analyzed by Western blotting with antibodies against γ-tubulin and Dgrips84 and 91. (E) The γ-tubulin complex has an S value of 9.5 S. The γ-tubulin complex isolated from Sf9 cells was sedimented on a 5–40% sucrose gradient. The resulting fractions were separated by 10% SDS-PAGE and stained by Coomassie blue. Protein standards with S values of 4.3 S (bovine serum albumin), 7.35 S (rabbit muscle aldolase), 11.3 S (bovine liver catalase), and 19.4 S (porcine thyroglobulin) were run on identical gradients. The peaks corresponding to these S values are indicated with arrowheads. (F) γ-Tubulin in the reconstituted γTuSC binds to guanine nucleotides. α-^{32}P-GTP was UV–cross linked to immunoisolated γTuSC in the absence (none) or presence of excess competing nonradioactive nucleotides as indicated. The proteins were separated by 10% SDS-PAGE and subjected to autoradiography.

$MgCl_2$, 1 EGTA. Homogenization buffer: HB100 plus 10% glycerol, 1 mM PMSF. Protease inhibitor stock: 10 mM benzamidine-HCl and 1 mg/ml each of aprotinin, leupeptin, and pepstatin A in ethanol. Flag peptide elution buffer: 0.5 mg/ml flag peptide (Sigma-Aldrich) in HB150.

Construction of Recombinant Baculovirus Expressing Dgrips and γ-Tubulin

Untagged γ-tubulin, 3′ Flag tagged γ-tubulin, and Dgrips84, 91, 128, and 163 were cloned individually into the pFastBac vector of the Bac-to-Bac baculovirus expression system (Life Technologies). The constructs were verified by sequencing and recombinant Baculoviruses were generated according to the manufacturer's instructions. A multiplicity of infection of 3–5 was used to coexpress the γTuRC subunits in Sf9 cells.

Purification of the Reconstituted γTuSC

Flag-tagged γ-tubulin and -untagged Dgrips84 and 91 were coexpressed in Sf9 cells and affinity purified using protein A–agarose beads precoupled to Flag antibody (Flag-M2 agarose beads; Sigma-Aldrich). Approximately 2–4 μg of total γ-tubulin complex could be isolated from ~10^8 Sf9 cells by this method. γTuSC was further purified on a 100-μl Mono S column run on a Smart System (Amersham Pharmacia Biotech) using a linear salt gradient generated between HB100 and HB1M as follows. The peptide-eluted γTuSC was loaded onto the Mono S column and eluted in 16 fractions of 100-μl each. 20 μl of the resulting fractions were run on a 10% SDS-PAGE gel and stained with Coomassie blue to determine the peak fraction of γTuSC.

GTP Cross Linking and Competition

γTuSC was immunoisolated with Flag antibody-coupled agarose beads, washed with buffer containing no GTP (see immunoprecipitation), and resuspended in 128 μl of BRB80. 16 μl of resuspended beads was incubated with 10 μCi of α-^{32}P-GTP alone or mixed with a 200-fold molar excess of unlabeled GTP, GDP, GTPγS, ATP, CTP, or UTP for 90 min on ice. The samples were UV cross linked for 5 min (Oegema et al., 1999), separated by SDS-PAGE, and analyzed by autoradiography.

Solution MT Nucleation Assay

A 4-mg/ml tubulin reaction mix was prepared by mixing unlabeled tubulin and rhodamine-labeled tubulin at a molar ratio of 6:1 in BRB80 with 1 mM GTP and 0.1% β-ME. 5 μl of the tubulin mix was added to 5 μl of sample (0.1–0.2 μM γTuSC or 0.5 mg/ml peptide) and incubated at 30°C for 5 min. MTs were fixed with 1% glutaraldehyde and the number of MTs was quantified by fluorescence microscopy as described previously (Zheng et al., 1995; Oegema et al., 1999).

MT Binding Experiments

Ethylene glycol bis-succinimidyl succinate (EGS) cross-linked MTs were prepared (Fanara et al., 1999) in the presence of biotinylated tubulin (7.2

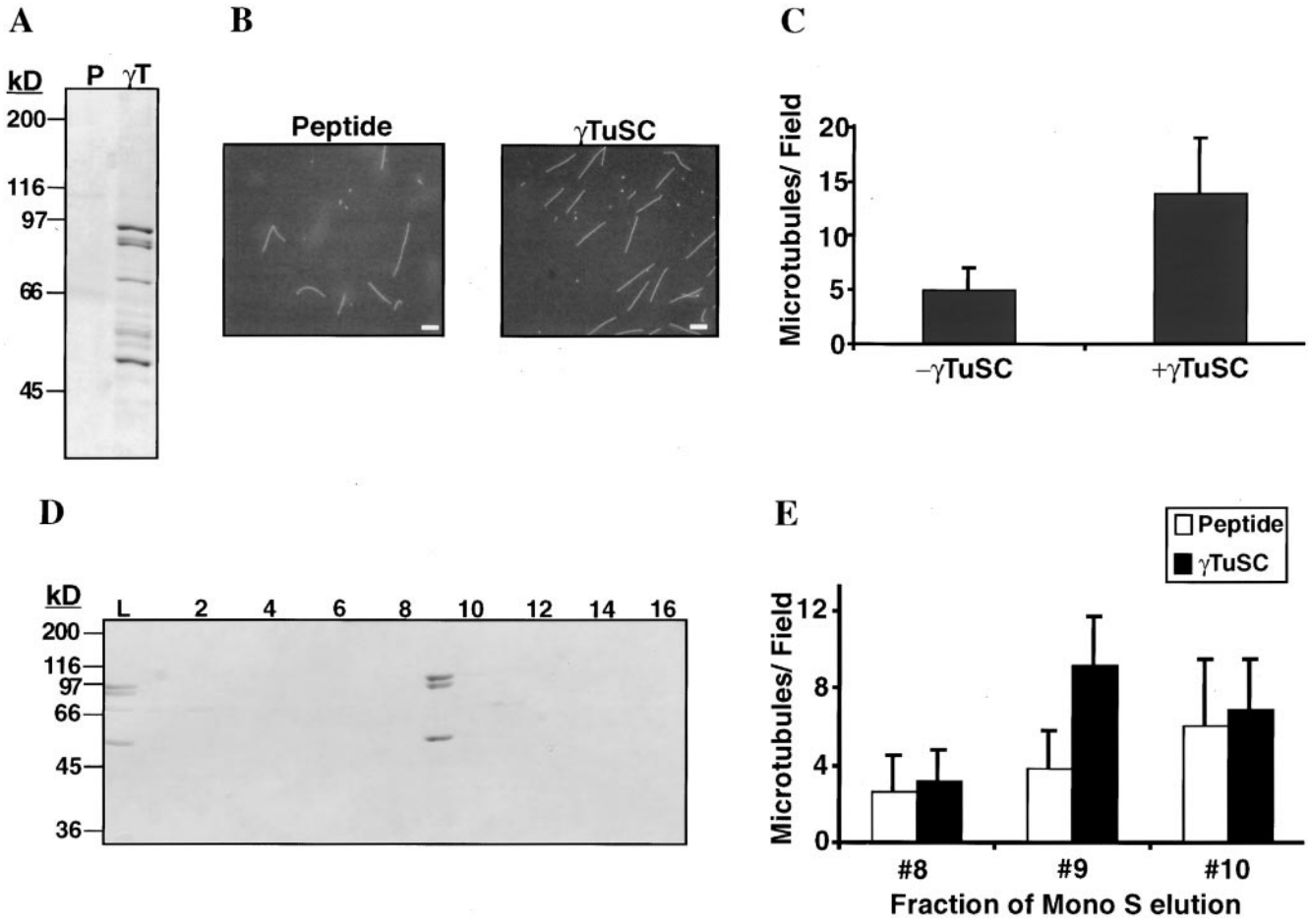

Figure 2. γTuSC nucleates MTs in vitro. (A) Coomassie blue stained 10% SDS-PAGE gel of peptide control (P) and γTuSC (γT) used for solution nucleation assays. (B) Images of representative fields of solution nucleation assays in the absence (peptide) or presence of γTuSC. Scale bar: 5 μm. (C) Comparison of the number of MTs nucleated in the absence (peptide) or presence of γTuSC from one representative experiment. The number of MTs in 30 random fields was counted and the average number of MTs per field was plotted. The error bars denote SD. (D) The immunoisolated γTuSC (L) was further purified by Mono S chromatography and the resulting fractions were analyzed on a 10% SDS-PAGE gel and stained with Coomassie blue. (E) MT nucleating activity of the γTuSC from the Mono S column as compared with corresponding fractions of a control Mono S purification of peptide alone (from one representative experiment). Only fraction nine, which contained the purified γTuSC, has significant MT nucleating activity compared with controls. MTs were counted and plotted as in C.

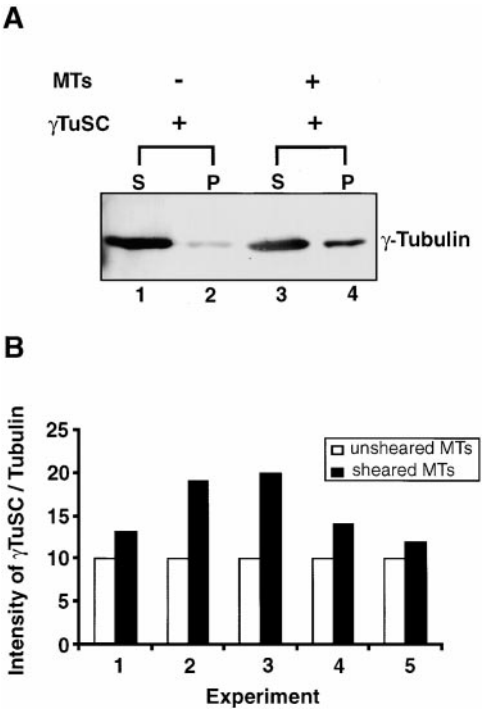

Figure 3. Reconstituted γTuSC binds to preformed MTs. (A) γTuSC was incubated in the absence (1 and 2) or presence (3 and 4) of EGS cross-linked and biotin-labeled MTs followed by incubation with streptavidin-linked magnetic beads. The supernatant (S, 1 and 3) and the beads (P, 2 and 4) were subjected to SDS-PAGE followed by Western blotting with antibodies against γ-tubulin to detect γTuSC. (B) γTuSC binds to the ends of MTs. γTuSC was incubated with either unsheared or sheared biotin-labeled taxol MTs. The MTs in each sample were retrieved with streptavidin-linked magnetic beads and analyzed by Western blotting with antibodies against α-tubulin (DM1α) to detect tubulin and γ-tubulin to detect γTuSC. The amount of γTuSC on the beads was normalized against the tubulin on the beads and plotted for each of the five experiments.

μM). The biotinylated-EGS-MTs were incubated with γTuSC (0.2 μM) for 15 min at 23°C followed by an additional 15 min of incubation with 5 μl of streptavidin-coupled magnetic beads preblocked with 1 mg/ml BSA (Dynal). γTuSC in the supernatant and the beads were analyzed by Western blotting with antibodies against γ-tubulin. The band intensity was quantified by densitometry and was within the linear range of the alkaline phosphatase detection system. To make taxol-stabilized biotinylated MTs, unlabeled tubulin and biotinylated tubulin (1:1 molar ratio; 2 mg/ml final) was prepared in the presence of 1 mM GTP, 1 mM DTT, and 10% DMSO in BRB80 and incubated at 37°C for 30 min. MTs were pelleted and used in the binding experiments. For MT shearing experiments, rhodamine-labeled tubulin (1:2:3 molar ratio of rhodamine, unlabeled, and biotinylated tubulins) was added into the nucleation reaction to visualize the MTs before and after shearing. The amount of tubulin and γTuSC on the beads was analyzed by Western blotting with antibodies against α-tubulin (DM1α) and γ-tubulin, respectively. The amount of γTuSC on the beads was normalized to the amount of tubulin on the beads to allow direct comparisons among different experiments.

MT Capping Assays

The "nucleation mix" was prepared by mixing unlabeled tubulin with rhodamine-labeled tubulin (2:1 molar ratio) in BRB80 with 1 mM GTP to a final tubulin concentration of 8 mg/ml. 3 μl of the tubulin mix was added to 3 μl of peptide (control) or purified γTuSC (0.1– 0.2 μM) and incubated at 37°C for 1 min. Then, 60 μl of prewarmed "elongation mix" containing unlabeled tubulin (1 mg/ml) in BRB80 containing 1 mM GTP and 0.1% β-ME was added to the nucleation mix and incubated for 5 min. 5 μl of the sample was fixed, photographed, and analyzed (Wiese and Zheng, 2000).

Drosophila Embryo Extract Preparation

Crude *Drosophila* embryo extract was prepared by homogenizing the embryos in homogenization buffer (Moritz et al., 1998), frozen in 3-ml aliquots in liquid nitrogen, and stored at −80°C. Extracts were clarified by centrifugation of the crude extract at 50,000 rpm in a TLS55 or TL100 rotor (Beckman) for 1 h at 4°C. γTuRC was purified as described (Oegema et al., 1999).

Cloning and Sequencing of Dgrips128 and 163

The Dgrips were isolated and the proteins were microsequenced as described previously (Oegema et al., 1999; Martin et al., 1998). Primers corresponding to peptides NLDDLAE and DELFTQFFA or KQRELQI and KTAASTSSGLHAEIPQDI were used to clone Dgrips128 and 163, respectively. Although the predicted molecular mass of the Dgrip128 cDNA clone was similar to the endogenous protein, there was no in frame stop codon preceding the start codon. However, in vitro translation of this Dgrip128 coding region produced a protein with the same size as the endogenous Dgrip128, suggesting that this cDNA is likely to encode the full-length Dgrip128. The Dgrip163 cDNA contained an in-frame stop codon preceding the start codon.

Sequence Analysis

Coiled-coil regions in Dgrips128 and 163 were predicted using the program MacStripe 2.0 (available online at http://www.york.ac.uk/depts/biol/units/coils/mstr2.html; Molecular Motors Group, Biology Department, University of York, York, UK). To define the grip motifs 1 and 2, Dgrips128 and 163 were used to search the database for proteins that share homologous regions using the BLASTP + BEAUTY Search program (BCM Search Launcher, General Protein Sequence/Pattern Search). After defining the grip motif regions in each of the grips, 9 and 10, sequences for grip motifs 1 and 2, respectively, were aligned using the multiple sequence alignment program ClustalW 1.8 (BCM Search Launcher, multiple sequence alignments).

Antibody Production and Western Blot Analysis

To generate rabbit polyclonal antibodies against Dgrips128 and 163, fusion protein constructs were made between glutathione-S-transferase (GST) and the first 200 amino acids of Dgrip128 or amino acids 590–811 of Dgrip163. The antibodies were affinity-purified against the corresponding fusion proteins after removal of GST antibodies. Western blotting was performed with affinity-purified antibodies at a concentration of ~1 μg/ml using either the ECL detection system (Amersham Pharmacia Biotech) or an alkaline phosphatase detection system (Promega Corp.).

Immunoprecipitations

Affinity-purified rabbit polyclonal antibodies against *Drosophila* γ-tubulin (DrosC), Dgrips84, 91 (Oegema et al., 1999), 128, or 163, or nonimmunized rabbit IgG (NR) were coupled to Affi-Prep– or agarose-protein A beads (Bio-Rad Laboratories). The antibody-bound beads were blocked with 1 ml of 10 mg/ml BSA or ovalbumin in HB100 for 1 h at 4°C with gentle rotation. The beads were washed three times with HB100 and incubated with either clarified *Drosophila* embryo extracts made from 0–3-h embryos or soluble Sf9 cell lysates from ~10^6 Sf9 cells infected with γTuRC subunits. After incubation for 1 h at 4°C, the beads were washed three times with HB100 plus 0.1% Triton X-100 followed by three washes with HB250 and HB100 for the *Drosophila* extracts. Three washes of HB500 were used instead of HB250 for the immunoprecipitations of Dgrips from Sf9 cell lysates. The immunoprecipitated proteins were analyzed by 10% SDS-PAGE and visualized either by Coomassie blue staining or Western blotting.

Sucrose Gradient Sedimentation

Sucrose gradients were prepared as described previously (Oegema et al., 1999). 100 μl of either *Drosophila* embryo extracts made from 0–3-h embryos or purified γTuSC reconstituted in Sf9 cells was loaded onto the gradient and centrifuged in a TLS55 rotor at 50,000 rpm in a Beckman ultracentrifuge for 2 h (*Drosophila* embryo extracts) or 4 h (purified γTuSC). The gradients were fractionated and analyzed (Oegema et al., 1999).

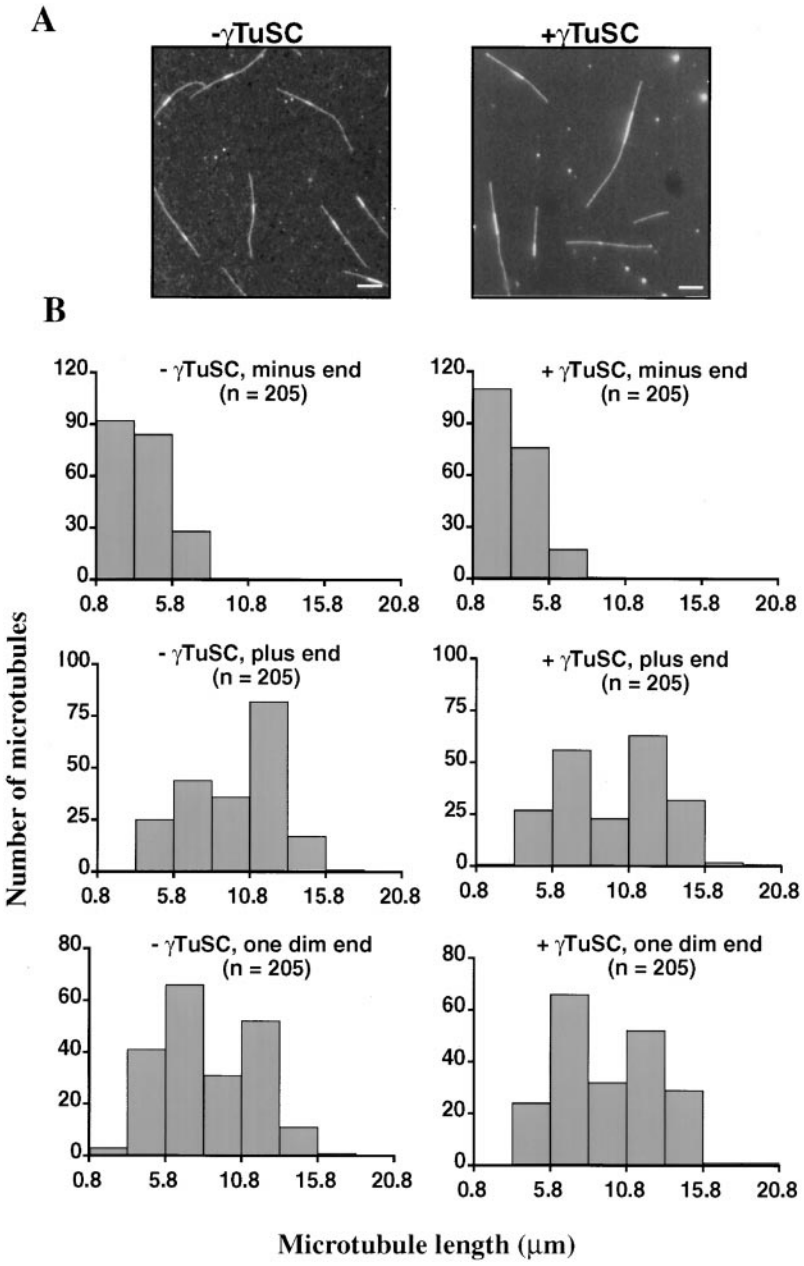

Figure 4. γTuSC caps the minus ends of MTs. (A) Representative fields of segmented MTs nucleated in the absence (peptide) or presence of γTuSC. Scale bar: 5 μm. (B) Length distributions of the segmented MTs. The two dim ends of "uncapped" MTs were measured in the presence of peptide control or reconstituted γTuSC. Length distributions for the minus and plus ends are shown (top and middle, respectively). (Bottom) Length distributions of the MTs that had only one dim end (capped MTs).

Embryo Fixation and Immunofluorescence

1–4-h *Drosophila* embryos were collected, fixed in methanol, and analyzed by immunofluorescence as described (Theurkauf, 1994). Embryos were labeled with a monoclonal antibody against γ-tubulin (GTU-88; Sigma-Aldrich) and rabbit antibodies against Dgrip163, followed by Alexa red–labeled anti–mouse and Alexa green–labeled anti–rabbit secondary antibodies (Molecular Probes). Images were obtained using a cooled CCD camera (Princeton Scientific Instruments, Inc.) on a Nikon E800 microscope and processed using Adobe Photoshop (Adobe Systems, Inc.).

In Situ Hybridization to the Drosophila Polytene Chromosomes

Nick-translated probes were prepared using biotin-16-2′-deoxyuridine-5′-triphosphate (bio-16-dUTP; ENZO diagnostics). Pretreatment and hybridization were previously described (Zhang and Spradling, 1994). Detection of biotin-labeled probes was performed using the Oncor chromosome in situ hybridization detection system (Oncor). DNA was stained with 4,6-diamino-2-phenylindole (DAPI) and propidium iodide and samples were mounted in Vectashield (Vector Laboratories). In situ hybridized chromosomes were examined by epifluorescence.

Results

Reconstitution, Purification, and Biochemical Characterization of the γTuSC

To understand γTuRC assembly and function, we first focused our attention on the γTuSC, a major subcomplex of the γTuRC. The *Drosophila* γTuSC is composed of three subunits: γ-tubulin and Dgrips84 and 91 (Oegema et al., 1999). To determine the nature of the interactions among these three proteins, γ-tubulin and Dgrip84, γ-tubulin and Dgrip91, or Dgrips84 and 91 were coexpressed in baculovirus. Reciprocal immunoprecipitations revealed that the three subunits interact with each other when expressed pair-

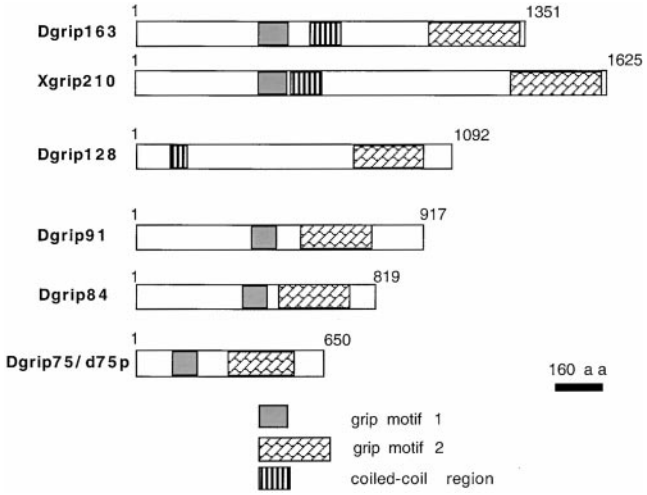

Figure 5. Schematic diagram of sequence motifs present in grips. Five grips from *Drosophila* and one grip from *Xenopus* are shown. The coiled-coil region was defined using the Macstripe 2.0b1f program (Lupas, 1997). The relative positions of the protein motifs shown are drawn to scale. Scale bar: ~160 amino acids.

wise (Fig. 1, A–C). Next, we coexpressed γ-tubulin and Dgrips84 and 91 in Sf9 cells and found that all three proteins coimmunoprecipitate with antibodies against any one of the subunits (data not shown). This data suggested that γ-tubulin and Dgrips84 and 91 had assembled into a complex.

To determine whether we had reconstituted the γTuSC, we purified the baculovirus expressed γ-tubulin complex using a Flag antibody against the Flag tagged γ-tubulin and analyzed its biophysical properties (Fig. 1 D). Hydrodynamic analysis showed that the reconstituted γ-tubulin complex had an S value of 9.5 S (Fig. 1 E) and a Stokes radius of ~7 nm, identical to the γTuSC purified from *Drosophila* embryos (Oegema et al., 1999). Using these two parameters, we estimated the reconstituted γ-tubulin complex has a molecular mass of ~270 kD (Siegel and Monty, 1966). In addition, based on densitometric analysis, the reconstituted γ-tubulin complex has an estimated stoichiometry of two γ-tubulin molecules per one molecule each of Dgrips84 and 91, which is identical to the stoichiometry of the endogenous γTuSC.

We previously showed that the γ-tubulin in the endogenous γTuSC binds guanine nucleotides, and that the γ-tubulin has a higher affinity for GDP than GTP (Oegema et al., 1999). Consistent with this observation, we found that the γ-tubulin in the reconstituted complex binds to guanine nucleotides with a higher affinity for GDP over GTP (Fig. 1 F). We also found that unlabeled ATP, CTP, and UTP did not compete with the guanine nucleotide in the γTuSC (Fig. 1 F). Taken together, these findings strongly suggest that we have reconstituted the γTuSC in Sf9 cells.

The Reconstituted Drosophila γTuSC Has MT Nucleating Activity In Vitro

To characterize the function of the reconstituted γTuSC, we investigated whether the reconstituted γTuSC could nucleate MTs in vitro. We immunoisolated the reconstituted γTuSC (Fig. 2 A) and found that it has a weak MT nucleating activity (Fig. 2, B and C), similar to that of endogenous γTuSC (Oegema et al., 1999). In 12 independent experiments, the reconstituted γTuSC at a concentration of 0.1–0.2 μM nucleated 2–10-fold more MTs than the peptide control with an average fold increase of 3.3 (Fig. 2, B and C). To confirm that the nucleation activity was due to the presence of the γTuSC, we further purified the immunoisolated γTuSC by Mono S anion exchange chromatography (Fig. 2 D). From three independent preparations of γTuSC, we found that the peak fraction of the Mono S purified γTuSC has 2.4–18.4-fold more MT nucleating activity than the control (Fig. 2 E), suggesting that the MT nucleating activity observed with the immunoisolated γTuSC is due to the γTuSC. Therefore, we used the immunoisolated γTuSC to perform the MT binding and capping experiments described below.

Table I. Analysis of MT Capping in the Absence or Presence of γTuSC

	−γTuSC			+γTuSC		
Experiment	No. capped	*n*	Percent capped	No. capped	*n*	Percent capped
1	25	406	6.2	89	436	20.4
2	13	532	2.6	41	612	3.7
3	253	1941	13	469	1681	28

Segmented MTs were prepared in the absence or presence of γTuSC and the number of MTs that were capped (with one dim end) were counted. The percentage of capped MTs was calculated from the total number (*n*) of segmented MTs counted for three independent experiments.

γTuSC Binds to Preformed MTs and Has an Affinity for MT Ends

Previous work has shown that γTuRC binds and caps the minus ends of MTs (Zheng et al., 1995; Wiese and Zheng, 2000). As the major component of the γTuRC, we hypothesized that γTuSC may interact with MTs in a similar manner. To test whether the reconstituted γTuSC binds to MTs, we incubated purified γTuSC with either EGS crosslinked or taxol-stabilized MTs (see Materials and Methods). We found that significantly more γTuSC copelleted with the preformed MTs compared with the control (Fig. 3 A), suggesting that γTuSC binds to MTs. We quantified the amount of γTuSC and tubulin in the pellet in three different experiments and found the molar ratio between γTuSC and tubulin to be ~1:6. To determine whether γTuSC binds to the ends or the sides of MTs, purified γTuSC was incubated with an equal amount of sheared or unsheared taxol-stabilized MTs. We found that 1.5–2-fold more γTuSC copelleted with the sheared MTs compared with the unsheared MTs (Fig. 3 B), suggesting that γTuSC binds to the ends of MTs.

γTuSC Has Weak MT Capping Activity

To test whether γTuSC caps the ends of MTs, we nucleated segmented MTs made in the absence or presence of γTuSC (Wiese and Zheng, 2000; Fig. 4 A). Since the plus end of a MT grows faster than its minus end, the long and short dim segments correspond to the plus and minus ends of the MT, respectively. We found that the length distribution of the plus and minus ends of MTs were similar in peptide control and γTuSC-containing samples (Fig. 4 B). However, in the presence of γTuSC (0.2 μM), there was a consistent increase (approximately threefold) in the number of MTs that

Figure 6. Sequence alignments of grip motifs. (A) Sequence alignments of grip motif 1. (B) Sequence alignments of grip motif 2. A consensus amino acid was defined when at least 70% of the sequences had identical or highly conserved amino acids (red). These amino acids are listed in red at the bottom of the multiple sequence alignments. Two positions in grip motif 2 were occupied by either W, F (blue), or L (green).

had only one dim segment (Table I). Based on the length distribution, the dim end of these "capped" MTs appears to be the plus end (Fig. 4 B, bottom), suggesting that the capped end is the minus end. These results suggest that the γTuSC can bind and cap the minus ends of MTs in vitro.

Taken together, we have shown that the reconstituted γTuSC has MT binding, capping, and nucleating activities that are characteristic of the γTuRC. However, compared with the γTuRC, γTuSC has weak activities in all three aspects. This is not surprising since multiple γTuSCs are required to form one γTuRC.

Identification of Dgrips128 and 163

To further understand the assembly of the γTuRC, we sought to identify the remaining components of the γTuRC and test whether these components can interact with γTuSC and allow γTuRC assembly. We purified the γTuRC and microsequenced its protein subunits (Oegema et al., 1999). Using the peptide sequence information, we cloned the cDNAs for Dgrips75, 128, and 163 by degenerate PCR and library screening. We found that Dgrip75, one of several proteins that migrates at ~75 kD, is identical to the recently described d75p (Fava et al., 1999). The sequences for Dgrips128 and 163 are deposited in the database under accession numbers AJ291604 and AJ291605, respectively. The genes encoding Dgrips75, 128, and 163 were mapped to *Drosophila* polytene chromosomes at 31F, 13C, and 68F, respectively, by in situ hybridization.

Dgrips128 and 163 Share Conserved Sequence Motifs with Previously Characterized Grips

BLAST sequence searches revealed that both Dgrips128 and 163 are novel proteins. However, Dgrip163 is homologous to the newly identified *Xenopus* γTuRC subunit,

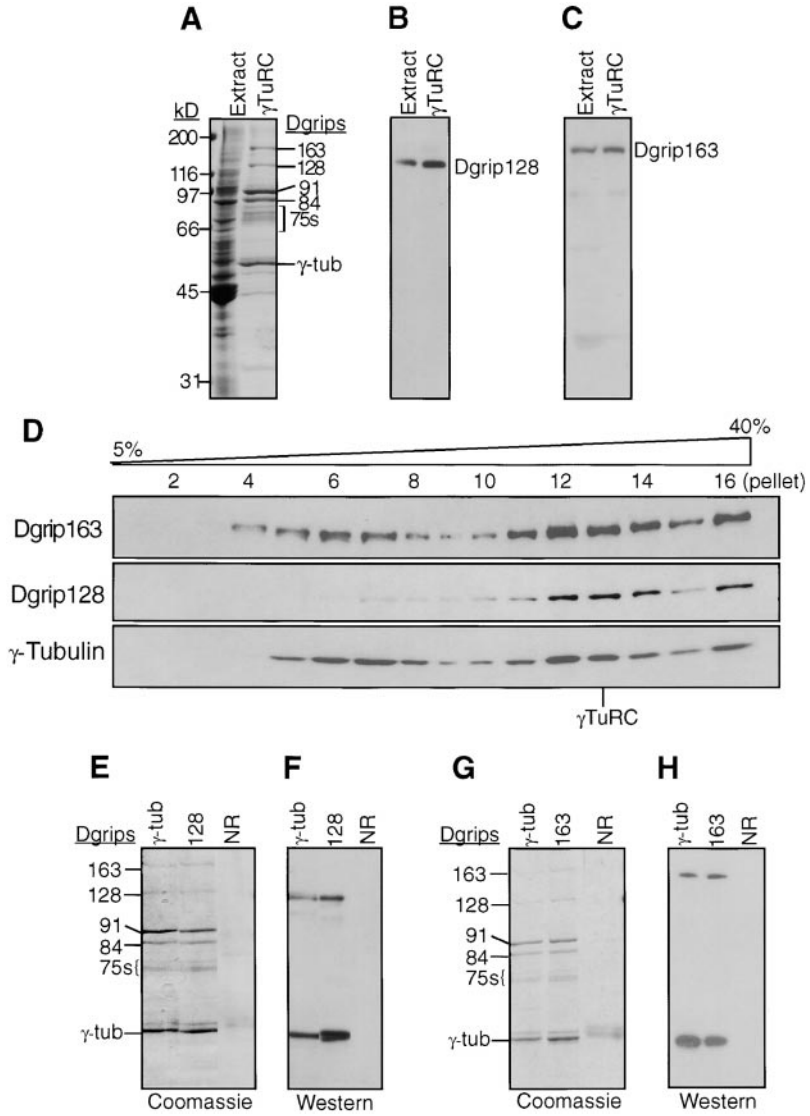

Figure 7. (A–C) Antibody specificity was tested by fractionating *Drosophila* embryo extract (Extract) or purified γTuRC (γTuRC) on a 10% SDS PAGE gel followed by Coomassie staining (A) or immunoblotting with antibodies against Dgrips128 (B) and 163 (C). (D) Clarified *Drosophila* embryo extract was sedimented through a 5–40% continuous sucrose gradient. The gradient fractions were analyzed by Western blotting with antibodies against γ-tubulin and Dgrips128 and 163, as indicated. (E–H) Antibodies against γ-tubulin (γ-tub), Dgrip128 (128), 163 (163), or random IgG (NR) were used to immunoprecipitate *Drosophila* embryo extract. The immunoprecipitates were analyzed on 10% SDS-PAGE gels followed by either Coomassie blue staining (E and G) or Western blotting (F and H), with antibodies against γ-tubulin and Dgrip128 (F) or γ-tubulin and Dgrip163 (H). The mouse monoclonal antibody against γ-tubulin (Sigma-Aldrich) used for Western blotting did not recognize the rabbit polyclonal antibodies (F and H, NR). γ-Tubulin migrates just below the heavy chain of the rabbit polyclonal antibodies (see the faint band above γ-tubulin in the Coomassie blue–stained gels).

Xgrip210 (Zhang et al., 2000), and both Dgrip163 and Xgrip210 are similar to a human partial expressed sequence tag (EST) (No. AL022328). Therefore, we suggest that Dgrip163 and Xgrip210 define a new family of conserved grips that also includes the putative Hgrip represented by the partial human EST.

Interestingly, we found that two subregions of Dgrip163 share significant sequence homology with Dgrips75, 84, and 91. We named these two homology regions grip motifs 1 and 2. Both motifs were present in all known grips with the exception of Dgrip128, which lacks grip motif 1. The spacing between the motifs varies among grips. In addition to grip motifs, Dgrips128 and 163 also contain a region predicted to form coiled-coil structures (Fig. 5).

To define the consensus sequences for each of the two grip motifs, we aligned known grips ranging from yeast to humans (see Materials and Methods) and found that grip motifs 1 and 2 are ~100 and 200 amino acids long, respectively (Fig. 6). Although the consensus sequences for both motifs are rich in leucine residues, they show no homology to each other. Moreover, the grip motifs do not appear to be similar to any previously reported sequence motifs, suggesting that they define two novel structural motifs unique to the grip family of proteins.

Dgrips128 and 163 Are Components of the γTuRC and Localize to the Centrosome

To characterize Dgrips128 and 163, we produced affinity-purified antibodies that were specific for each protein (Fig. 7, A–C). Sucrose density gradient sedimentation of *Drosophila* embryo extracts showed that Dgrips128 and 163 cofractionated with γ-tubulin (Fig. 7 D). Although some Dgrip163 remained at the top of the sucrose gradient, we found that the majority comigrated with Dgrip128 and γ-tubulin in fractions 12–14. In addition, we found that antibodies against γ-tubulin and Dgrips128 and 163 immunoprecipitated the same set of γTuRC proteins from *Drosophila* embryo extracts (Oegema et al., 1999) (Fig. 7, E–H). Together, these results suggest that Dgrips128 and 163 are components of the *Drosophila* γTuRC.

To determine whether Dgrips128 and 163 are centrosomal proteins, we performed immunofluorescence of *Drosophila* embryos with antibodies against γ-tubulin and Dgrips128 and 163. We found that Dgrip163 colocalized with γ-tubulin at the centrosome in interphase and mitosis (Fig. 8). Interestingly, like γ-tubulin, a fraction of Dgrip163 also localized to the mitotic spindles. Antibodies against Dgrip128 produced no signal by immunofluores-

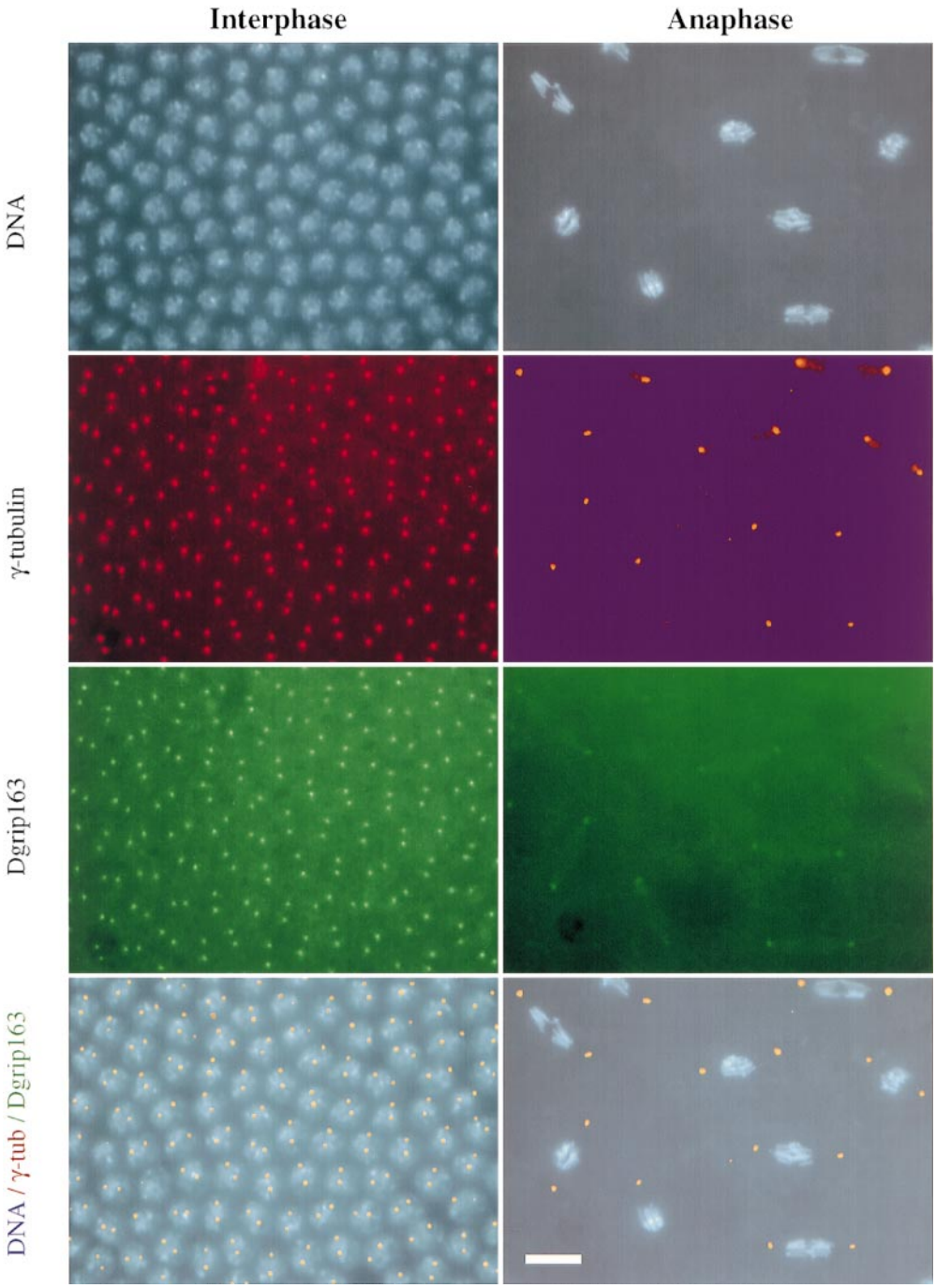

Figure 8. Dgrip163 colocalizes with γ-tubulin at the centrosomes. Immunofluorescence staining of early *Drosophila* embryos with antibodies against Dgrip163 (green) and γ-tubulin (red). Both proteins localize strongly to the centrosomes and weakly to the spindles. Examples of embryos in interphase and anaphase are shown for each antibody. Scale bar: 15 μm.

cence. However, we found that Dgrip128 was enriched in isolated *Drosophila* centrosomes (Moritz et al., 1995b) by Western blotting analysis with antibodies against Dgrip128 (data not shown). This suggested that Dgrip128 is also a centrosomal protein.

Dgrips128 and 163 Interact with γTuSC but Do Not Assemble into a γTuRC

To determine whether Dgrips 128 and 163 interact with γTuSC, either Dgrip128 or Dgrip163 was coexpressed in the baculovirus system with γTuSC and analyzed by immunoprecipitation. We found that both Dgrips128 and 163 interacted with γTuSC individually (Fig. 9). Next, we coexpressed Dgrips128 and 163 with γTuSC to determine whether we could reconstitute the γTuRC in the baculovirus system. Although all five subunits were coexpressed, we found that γ-tubulin and Dgrip84 and 91 comigrated on sucrose gradients at a position expected for the γTuSC, but not the γTuRC (data not shown). These results indicated that the interactions between Dgrip128 or Dgrip163 and γTuSC are insufficient for the assembly of the γTuSC into γTuRC. Therefore, the remaining unidentified γTuRC components are most likely necessary for γTuRC assembly.

Discussion

Since MTs are central to many cellular functions, it is important to understand their nucleation in molecular detail. Although previous studies have begun to reveal the structure and composition of the γTuRC, many important

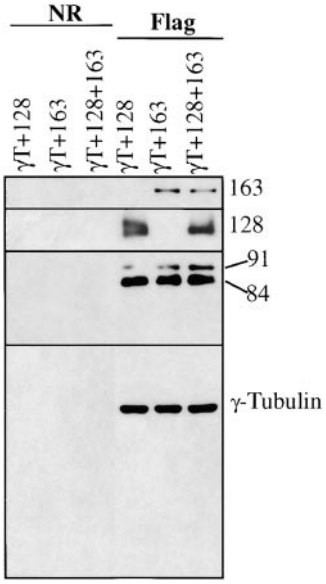

Figure 9. Dgrips128 and 163 coimmunoprecipitate with γTuSC. Dgrips128 and/or 163 were coexpressed with γTuSC in Sf9 cells and immunoprecipitated with either control (NR) or Flag (Flag) antibodies against the Flag tagged γ-tubulin in γTuSC. The immunoprecipitated proteins were separated on a 10% SDS gel and analyzed by Western blots with antibodies against all five γTuRC subunits. The proteins that were coexpressed are indicated at the top of the gel.

questions still remain. For example, little is known about the assembly of this multisubunit complex. It is also unclear how the γTuRC is recruited and tethered to the centrosome. The studies presented here provide important clues to some of these questions.

Reconstitution of the γTuSC

With the reconstitution of the γTuSC, which is a major subcomplex of γTuRC, we have made an important step toward understanding the assembly and function of the γTuRC. By both functional and biochemical criteria, the reconstituted γTuSC is identical to the endogenous γTuSC. In addition to its nucleating activity, we showed, for the first time, that the γTuSC can also bind and cap the minus ends of MTs. This observation is consistent with the finding that the monomeric γ-tubulin (Li and Joshi, 1995; Leguy et al., 2000) and γTuRC (Zheng et al., 1995; Wiese and Zheng, 2000) can also bind and cap the minus ends of MTs. Compared with γTuRC, γTuSC is a weak MT nucleator (Oegema et al., 1999; this study). Consistent with its weak nucleating activity, we found that the capping activity of γTuSC is significantly less than that of the γTuRC. For example, ~50% of MTs nucleated in the presence of γTuRC were capped (Zheng et al., 1995; Wiese and Zheng, 2000), while under the same assay conditions, γTuSC could only cap up to 20% of MTs. One explanation for this weak nucleating and capping activity of γTuSC is that γTuSC contains only two γ-tubulin molecules, while the intact γTuRC contains ~12 γ-tubulin molecules.

The Grip Motifs in γTuRC Assembly and Recruitment

The existence of the conserved sequence motifs in all five of the grips suggests that γTuRC assembly may be mediated by conserved structural surfaces defined by these motifs. A provocative idea is that grip motif 2, which is present in all five grips, is involved in interacting with a common protein in the γTuRC (e.g., γ-tubulin). Consistent with these ideas, we observed that γ-tubulin coimmunoprecipitated with each of the five Dgrips when coexpressed in pairs (our unpublished observations). Furthermore, using similar methods, we found that the Dgrips also interacted with each other (unpublished observations). It will be important to study the nature of these interactions and test whether they are mediated by the grip motifs.

Alternatively, the grip motifs could be involved in binding the γTuRC to its centrosomal docking site. Using in vitro centrosome assembly assays in *Xenopus* egg extracts, we have shown that the removal of Xgrip210 (Dgrip163 homologue) blocks the localization of Xgrip109 (Dgrip91 homologue) to the centrosome and vice versa (Zhang et

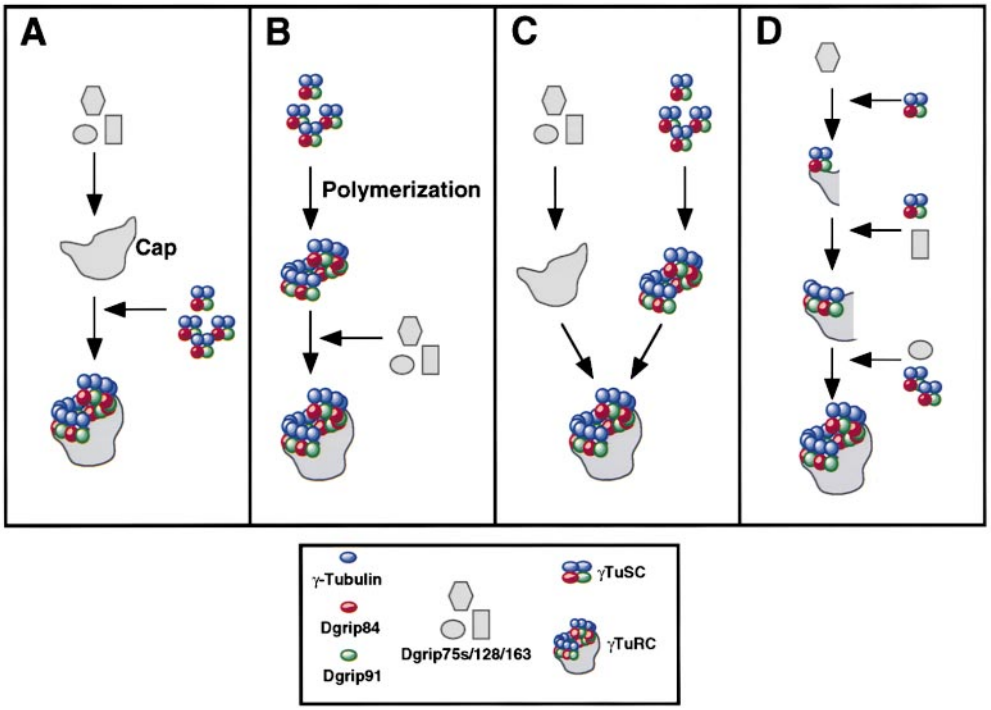

Figure 10. Four models for γTuRC assembly. (A) Scaffold model in which the non–γTuSC subunits preassemble to form a cap that acts as a scaffold that binds γTuSCs. (B) Polymerization model in which the self polymerization of γTuSC drives γTuRC assembly. (C) Oligomer-capping model in which both γTuSC polymerization and preassembly of the cap are required. (D) Sequential model in which the interaction between certain γTuRC subunits triggers a cascade of events leading to γTuRC assembly.

al., 2000). This suggests that the grip motifs present in Xgrips109 and 210 are not sufficient for the binding of individual grips to the centrosome. Therefore, it is unlikely that the individual grip motifs per se are sufficient to mediate the recruitment and binding to the centrosomes. Instead, a certain combination of the grip motifs may be required for centrosomal docking to take place, and such a structural surface may only occur in the intact γTuRC.

The γTuRC Assembly Pathway

Based on the structural features of the γTuRC (Moritz et al., 2000), we propose four possible models for γTuRC assembly. In the first model, the cap structure of the γTuRC (Moritz et al., 2000) acts as a scaffold onto which multiple γTuSCs assemble to form a ring (Fig. 10 A). In this assembly pathway, the formation of the ring requires preassembly of the cap structure. In the second model, multiple γTuSCs oligomerize and individual cap subunits add onto this oligomer to form the γTuRC (Fig. 10 B). In this model, prior assembly of a cap structure is not required and γTuSC polymerization drives the assembly process. The third model features the preassembly of both a cap structure and γTuSC oligomers. In this model, the γTuSC oligomers are stabilized by the preformed cap structure to form a γTuRC (Fig. 10 C). Finally, the fourth model predicts that the γTuRC is assembled sequentially from several distinct intermediates (Fig. 10 D).

The majority of the reconstituted and purified γTuSC migrated as a 10 S complex on sucrose gradients. However, a small fraction of the γTuSC appears to oligomerize and migrate faster than the 10 S complex (Gunawardane, R.N., and Y. Zheng, unpublished observation). This observation suggests that oligomerization of γTuSC could contribute toward γTuRC assembly. Our success in γTuSC reconstitution should allow us to further test conditions that promote γTuSC oligomerization and aid the study of γTuRC assembly.

Although coexpressing Dgrips128 and 163 with γTuSC did not promote γTuSC oligomerization or γTuRC assembly, both these proteins can interact with γTuSC independent of each other. These interactions may give rise to the assembly intermediates, as suggested by the sequential pathway of γTuRC assembly (Fig. 10 D). In addition, in vitro assays using *Xenopus* egg extract showed that the Dgrip163 homologue Xgrip210 is essential for γTuRC assembly (Zhang et al., 2000). Based on these observations, we suggest that Dgrips128 and 163 are essential but not sufficient for γTuRC formation. If all γTuRC subunits are needed for its assembly, the identification and expression of Dgrip75s (the remaining subunits of γTuRC) should permit the reconstitution of the γTuRC and the testing of the various models for γTuRC assembly.

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