Characterization of a New γ TuRC Subunit with WD Repeats

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The γ -tubulin ring complex (γ TuRC), consisting of multiple protein subunits, can nucleate microtubule assembly. Although many subunits of the γ TuRC have been identified, a complete set remains to be defined in any organism. In addition, how the subunits interact with each other to assemble into γ TuRC remains largely unknown. Here, we report the characterization of a novel γ TuRC subunit, *Drosophila* gamma ring protein with WD repeats (Dgp71WD). With the exception of γ -tubulin, Dgp71WD is the only γ TuRC component identified to date that does not contain the grip motifs, which are signature sequences conserved in γ TuRC components. By performing immunoprecipitations after pair-wise coexpression in Sf9 cells, we show that Dgp71WD directly interacts with the grip motif-containing γ TuRC subunits, Dgrips84, 91, 128, and 163, suggesting that Dgp71WD may play a scaffolding role in γ TuRC organization. We also show that Dgrips128 and 163, like Dgrips84 and 91, can interact directly with γ -tubulin. Coexpression of any of these grip motif-containing proteins with γ -tubulin promotes γ -tubulin binding to guanine nucleotide. In contrast, in the same assay Dgp71WD interacts with γ -tubulin but does not facilitate nucleotide binding.

INTRODUCTION

γ -Tubulin is a ubiquitous and highly conserved centrosomal protein that is essential for microtubule (MT) function (Wiese and Zheng, 1999; Oakley, 2000). Early studies showed that disruption of γ -tubulin function resulted in various defects in MT assembly and lethality to the cell (Oakley and Oakley, 1989; Oakley *et al.*, 1990; Horio *et al.*, 1991; Joshi *et al.*, 1992; Sobel and Synder, 1995; Sunkel *et al.*, 1995). This led to the hypothesis that γ -tubulin is involved in microtubule nucleation at the centrosomes. Recent studies of γ -tubulin in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Aspergillus nidulans* suggest that γ -tubulin is also involved in MT organization and spindle assembly (Paluh *et al.*, 2000; Prigozhina *et al.*, 2001; Strome *et al.*, 2001). Therefore, γ -tubulin appears to have roles in MT organization in addition to regulating microtubule nucleation.

Higher eukaryotes contain centrosomal γ -tubulin and a significant pool of cytosolic γ -tubulin in the form of large protein complexes (Wiese and Zheng, 1999). The cytosolic

γ -tubulin does not appear to exist as monomers in organisms examined so far, suggesting that it does not function alone. One γ -tubulin containing complex, the γ -tubulin ring complex (γ TuRC), has been purified from *Xenopus*, *Drosophila*, and humans (Zheng *et al.*, 1995; Oegema *et al.*, 1999; Murphy *et al.*, 2001). γ TuRCs from these organisms share a similar structure and protein composition, and they can nucleate MTs in vitro (Zheng *et al.*, 1995; Oegema *et al.*, 1999; Murphy *et al.*, 2001). Additional biochemical and structural studies reveal that γ TuRC is recruited to the centrosome to mediate MT nucleation (Moritz *et al.*, 1995a, 1995b; Martin *et al.*, 1998; Schnackenberg *et al.*, 1998).

In addition to the γ TuRC, the cytosolic γ -tubulin in *Drosophila* embryos and *Xenopus* eggs exists in a smaller complex known as the γ -Tubulin Small Complex (γ TuSC; Oegema *et al.*, 1999; Zhang *et al.*, 2000). The *Drosophila* γ TuSC is a tetramer of ~ 10 S composed of two γ -tubulin molecules and one each of Dgrips84 and 91 (Oegema *et al.*, 1999). The γ TuSC subunits are the most abundant proteins of the γ TuRC and are thought to form the functional and structural core of the γ TuRC (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000). In addition, the γ TuSC appears to be the most conserved unit of the γ TuRC because its homologues show significant sequence conservation across species (Wiese and Zheng, 1999; Gunawardane *et al.*, 2000). Indeed, the Tub4p complex, the cytosolic γ -tubulin complex found in *Saccharomyces cerevisiae*, is similar in size and composition to *Drosophila* γ TuSC (Geissler *et al.*, 1996; Knop and Schiebel, 1997; Vinh *et al.*, 2002). Whereas the Tub4p of the Tub4p complex

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Abbreviations used: γ TuRC, γ -tubulin ring complex; γ TuSC, γ -tubulin small complex; Dgrip, *Drosophila* gamma ring protein; β -ME, β -mercaptoethanol; MT, microtubule; Dgp71WD, *Drosophila* gamma ring protein of 71 kDa with WD repeats; NR, nonimmunized rabbit IgG.

is a homolog of γ -tubulin, each of the other two subunits of the Tup4p complex, Spc97p and Spc98p, share significant homology to Dgrips84 and 91, respectively (Geissler *et al.*, 1996; Knop and Schiebel, 1997; Oegema *et al.*, 1999; Vinh *et al.*, 2002).

To date five human and five *Drosophila* γ TuRC subunits in addition to γ -tubulin have been identified (Murphy *et al.*, 1998; Fava *et al.*, 1999; Oegema *et al.*, 1999; Gunawardane *et al.*, 2000; Murphy *et al.*, 2001). Sequence analysis of these γ TuRC subunits revealed that they share regions of sequence conservation (Gunawardane *et al.*, 2000; Murphy *et al.*, 2001), which we named grip motifs 1 and 2 (Gunawardane *et al.*, 2000). Electron microscopy (EM) analysis of the *Drosophila* γ TuRC (Oegema *et al.*, 1999; Moritz *et al.*, 2000) showed that the complex consists of two major substructures: the γ TuRC ring composed of repeating columnar units (Oegema *et al.*, 1999; Moritz *et al.*, 2000) and a cap structure that covers one face of the γ TuRC ring (Moritz *et al.*, 2000). Based on the EM analyses and biochemical characterization of the *Drosophila* γ TuRC (Oegema *et al.*, 1999), a structural model for the γ TuRC was proposed (Moritz *et al.*, 2000). According to this model, the modular subunits of the γ TuRC ring correspond to multiple γ TuSCs and the cap structure is composed of Dgrips75s, 128, and 163 (Oegema *et al.*, 1999; Moritz *et al.*, 2000). Indeed, Xgrip210, the *Xenopus* homolog of Dgrip163, is localized to the cap structure of the purified *Xenopus* γ TuRC by immunogold EM (Keating and Borisy, 2000; Zhang *et al.*, 2000).

Although the above γ TuRC model is appealing, how the subunits interact with each other to assemble into γ TuRC remains largely unknown. For example, it is not clear whether the ring of the γ TuRC is made of the γ TuSCs exclusively. It is also not clear why the subunits found in the cap structure such as Dgrip128 and Dgrip163 contain the conserved grip motifs. Because the two grip motif-containing subunits, Dgrip84 and Dgrip91, interact with γ -tubulin, an important question is whether γ -tubulin also interacts with the other subunits in the γ TuRC that contain the grip motifs. The identification of a new γ TuRC subunit, Dgp71WD, that does not contain grip motifs allowed us to address some of these important questions.

MATERIALS AND METHODS

Buffers and Reagents

H buffer: 50 mM HEPES, pH 7.4, 1 mM MgCl_2 , 1 mM EGTA, 1 mM β -mercaptoethanol (β -ME), 0.1 mM GTP, and protease inhibitor stock at 1:200 final dilution as previously described (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000). H100, H150, and H500: H buffer plus 100 mM, 150 mM, or 500 mM NaCl, respectively. Protease inhibitor stock: 10 mM benzamidinium-HCl and 0.1 mg/ml phenanthroline, and 1 mg/ml each of aprotinin, leupeptin, and pepstatin A in ethanol. Flag M2 mAb coupled to agarose beads was used to immunoprecipitate the γ TuRC subunits that were tagged with Flag (Sigma, St. Louis, MO). Antibodies against γ -tubulin, Dgrips84, 91, 128, and 163 were described previously (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000).

Cloning Dgp71WD

Database searches using internal peptides obtained by microsequencing allowed us to identify a partial EST clone that matched some of the peptide sequences. However, when sequenced, this EST clone did not contain a complete open reading frame (ORF). Using

the EST sequence and the sequenced *Drosophila* genome, we identified a gene whose predicted protein sequence matched 10 of the peptides (KTVLSDFADLE, KTPEIQ, KQSLE, KSEYV, KEFSEL, DFVDQ, KAPLAVR, DEYIAAV, DAAVTRVAFVPVP, IAAANLLS). By excising the intervening introns we obtained the full-length coding sequence for a putative γ TuRC subunit whose sequence was further confirmed by GENSCAN (www.genes.mit.edu/GENSCAN.html). The full-length cDNA was then cloned using *Drosophila* embryonic mRNA by RT-PCR and subcloned into the pFAST-BAC1 vector (Invitrogen, Carlsbad, CA) for baculovirus expression. Using the Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl), we found that this protein contained five WD repeats. We refer to this protein as *Drosophila* gamma ring protein of 71 kDa with WD repeats (Dgp71WD).

Antibody Production and Purification

Three peptides, EKDGKTPEIQRV (aa 59–70), DWETLNRKPOPY-ETANRQS (aa 399–417), and ENEMLKAKLKFYQEQQETES (aa 624–643) of Dgp71WD were synthesized and used for antibody production (Zymed, San Francisco, CA). Only one peptide, DWETLNRKPOPYETANRQS (aa 399–417), induced an immune response in rabbits. The antibodies were affinity-purified against the peptide coupled to Sulfo-Link resin (Pierce, Rockford, IL). Antibody against the C-terminal peptide of γ -tubulin (DrosC) was previously described (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000). Antibody against the full-length maternal form of *Drosophila* γ -tubulin (D γ 2) was raised in rabbit and affinity-purified.

Immunofluorescence

One- to 2-h *Drosophila* embryos were fixed in methanol and examined by immunofluorescence (Theurkauf, 1992). Double labeling was performed with a mouse mAb against human γ -tubulin (Sigma) and rabbit antibody against *Drosophila* Dgp71WD, followed by Alexa Red or Green secondary antibodies (Molecular Probes, Eugene, OR). Images were obtained using a cooled CCD camera (Princeton Scientific Instruments, Inc., Monmouth Junction, NJ) on a Nikon E800 microscope and processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Expression and Immunoprecipitation of Proteins

Baculoviruses containing Flag-tagged or untagged γ -tubulin, Dgrips84, 91, 128, 163, and Dgp71WD were used to infect 100 ml cultures of 2×10^6 /ml Sf9 cells in different combinations. Each virus was infected at an MOI of ~ 3 and the cells were harvested 48–72 h postinfection. Soluble cell lysates were prepared in H150 by sonication (Gunawardane *et al.*, 2001) and centrifugation at 50,000 rpm for 10 min in a TLA120 rotor (Beckman, Palo Alto, CA). The lysates were used for immunoprecipitation with either antibodies against each protein or with Flag-M2 agarose beads (Sigma). For immunoprecipitations, antibodies against the γ TuRC subunits were first bound to protein A Affiprep beads (Bio-Rad) for 1 h at room temperature. BSA, 1–2 mg/ml, was used to block the antibody bound beads as well as Flag-M2 beads for at least 2 h at 4°C. After blocking, the beads were washed with H150 and then added to the cell lysates. The lysates were rotated for 1–2 h at 4°C to allow binding of the antibodies to proteins. After incubation, the beads were washed three times each with H150, followed by H500 and then by H150. The proteins that bound to the beads were separated by SDS-PAGE and analyzed by either Coomassie Blue staining or by Western blotting.

GTP Cross-linking

The GTP binding properties of γ -tubulin were assayed as described (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000). Flag-tagged γ -tubulin was expressed alone or coexpressed pair-wise with untagged Dgrips84, 91, 128, 163, or Dgp71WD in 2×10^8 Sf9 cells. The proteins

Figure 1. Dgp71WD contains five putative WD repeats. (A) Subunit composition of the *Drosophila* γ TuRC. *Drosophila* γ TuRC was immunoprecipitated from *Drosophila* embryo extracts with a peptide antibody against γ -tubulin cross-linked to protein A beads. The immunoprecipitates were separated on a 10% SDS gel and stained with Coomassie Blue (A). The group of proteins with apparent molecular masses of ~ 75 kDa is referred to as Dgrip75s and is indicated with a bracket. (B) The amino acid sequence of Dgp71WD. The microsequenced peptides are shown in boxes. (C) Sequence alignment of the five putative WD repeats in Dgp71WD, Neddl1, and COP1 using Clustal W.

in the soluble cell lysates were immunoprecipitated with 50 μ l of settled Flag-M2 agarose beads, washed with buffer containing no GTP, and resuspended in 128 μ l BRB80 (80 mM K-PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF). Sixteen microliters of resuspended beads was incubated with or without 10 μ Ci of α - 32 P-GTP (Amersham Pharmacia Biotech Inc.) for 90 min on ice. The samples were then UV cross-linked using a Stratalinker UV (Stratagene, La Jolla, CA) for 5 min, separated by 10% SDS-PAGE, and analyzed by autoradiography.

Sucrose Gradient Sedimentation Analysis of Proteins

Linear gradients of 5–40% sucrose were prepared in H100 with 0.1 mM GTP and 1:200 protease inhibitor stock as previously described

(Gunawardane *et al.*, 2001). Zero- to 2-h fly embryo extracts were fractionated on the gradients as described (Oegema *et al.*, 1999).

RESULTS

Dgp71WD Is a Novel γ TuRC Subunit that Contains WD repeats

The *Drosophila* γ TuRC consists of ~ 8 polypeptides of which γ -tubulin and Dgrips75 (76p), 84, 91, 128, and 163 have been identified and characterized (Fava *et al.*, 1999; Oegema *et al.*, 1999; Gunawardane *et al.*, 2000; also see Figure 1A). Interestingly, all of these Dgrips contain conserved grip motifs (Gunawardane *et al.*, 2000). On the basis of the protein

profile of the purified *Drosophila* γ TuRC, we estimated that two to three additional Dgrips with apparent molecular masses of ~ 75 kDa remained to be cloned and characterized (Figure 1A). Through database searches, we found that several peptide sequences that we obtained by microsequencing of the purified γ TuRC matched a *Drosophila* partial EST (see MATERIALS AND METHODS). We cloned the full-length cDNA (Figure 1B) and found that it contained five WD repeats (Figure 1C). Therefore, we refer to this novel protein as Dgp71WD, which stands for *Drosophila* gamma ring protein of 71 kDa with WD repeats (Dgp71WD, accession number AF461267). Interestingly, Dgp71WD did not contain the grip motifs found in all of the other Dgrips identified thus far. Database searches revealed a large number of proteins from various organisms sharing sequence homology with the WD-repeat regions of Dgp71WD. The alignment of the five WD repeats in Dgp71WD with the WD repeats of the *Arabidopsis* photomorphogenesis repressor COP1 (accession number A44272; Holm *et al.*, 2001) and the mouse Nedd1 (accession number P33215; Kumar *et al.*, 1992) is shown (Figure 1C). Interestingly, the presently uncharacterized mouse Nedd1 is similar in size to Dgp71WD and both proteins have the WD repeats at their N-termini. Pair-wise alignment (GCG Lite at NIH) showed that the overall amino acid identity between Nedd1 and Dgp71WD is 20.8% and that the homology between the two proteins extends beyond the WD repeats. Further studies of Nedd1 will be needed to determine whether it is a mouse homolog of Dgp71WD.

To determine if Dgp71WD is a γ TuRC subunit, rabbit polyclonal antibodies against the C-terminal peptide of γ -tubulin (DrosC; Oegema *et al.*, 1999) or against a peptide corresponding to amino acids 399–417 of Dgp71WD were used to immunoprecipitate Dgp71WD from *Drosophila* embryo extract. We found that anti-Dgp71WD antibody immunoprecipitated the same set of γ TuRC subunits as did the anti- γ -tubulin antibody, suggesting that Dgp71WD was a subunit of the γ TuRC. Furthermore, the antibody against Dgp71WD recognized a protein of ~ 71 kDa in γ TuRC immunoprecipitated with γ -tubulin antibodies (Figure 2, A and B). In addition, when *Drosophila* embryo extracts were subjected to sucrose gradient sedimentation, Dgp71WD comigrated with the other γ TuRC subunits (Figure 2C). Because the previously characterized γ TuRC subunits localize to the centrosome throughout the cell cycle, we tested if Dgp71WD showed a similar localization pattern. We found that Dgp71WD colocalized with γ -tubulin at centrosomes in *Drosophila* embryos during interphase and mitosis (Figure 2, D and E, respectively). Taken together, these results showed that Dgp71WD is a subunit of the *Drosophila* γ TuRC.

Dgp71WD Directly Interacts with the Grip Motif-containing γ TuRC Subunits Dgrips84, 91, 128, and 163

Because Dgp71WD contains WD repeats and no grip motifs, we reasoned that it might play a scaffolding role by binding to the grip motif-containing subunits. If this is true, Dgrips84, 91, 128, and 163 should directly interact with Dgp71WD. To test this possibility, we coexpressed Dgp71WD with each of the Flag-tagged Dgrips84, 91, 163, or untagged Dgrip128, in Sf9 cells. Specific antibodies that recognize Dgp71WD, Dgrips84, 91, 128, 163, or Flag antibody

were used to immunoprecipitate each of the proteins. Non-immunized rabbit IgG was used as a control for each set of immunoprecipitations. The immunoprecipitates were first separated by SDS-PAGE and the proteins were either stained by Coomassie Blue or probed by Western blotting with specific antibodies. We found that the Dgp71WD antibody immunoprecipitated Dgrips84, 91, 128, and 163 (Figure 3, A–D). Conversely, antibodies that immunoprecipitated each of the Dgrips also immunoprecipitated Dgp71WD (Figure 3, A–D). On the basis of these results, we conclude that Dgp71WD interacts with each of the four grip-motif-containing γ TuRC subunits.

Dgp71WD Interacts with γ -Tubulin

Next, we asked whether Dgp71WD also directly interacts with γ -tubulin. We coexpressed Dgp71WD and γ -tubulin in Sf9 cells and used antibodies against each of the proteins for immunoprecipitation. We found that although the antibody against γ -tubulin immunoprecipitated Dgp71WD, the antibody against Dgp71WD did not immunoprecipitate γ -tubulin (Figure 4, A and B). The lack of reciprocal immunoprecipitation could reflect a lack of interaction between γ -tubulin and Dgp71WD. Alternatively, it is possible that Dgp71WD and γ -tubulin do interact with each other, but the binding of this particular antibody to Dgp71WD disrupts this interaction. The disruption is possible if the Dgp71WD antibody bind to the region of Dgp71WD involved in its binding to γ -tubulin.

We reasoned that if γ -tubulin and Dgp71WD do interact with each other, we should be able to show this interaction using different antibodies against γ -tubulin that do not interfere with this interaction. We expressed Dgp71WD and γ -tubulin either alone or together in Sf9 cells. Western blotting showed that similar amounts of Dgp71WD or γ -tubulin were expressed under either individual-expression or coexpression conditions (Figure 4C). We used the DrosC and D γ 2 antibodies raised against the C-terminal peptide of γ -tubulin and the full-length γ -tubulin, respectively, to immunoprecipitate γ -tubulin. As controls, nonimmunized rabbit IgG (NR) and the antibody against Dgp71WD were used. We found that NR did not immunoprecipitate γ -tubulin or Dgp71WD as expected (Figure 4D). Consistent with the earlier result (Figure 4, A and B), the Dgp71WD antibody did not immunoprecipitate γ -tubulin even when both Dgp71WD and γ -tubulin were coexpressed (Figure 4D). Importantly, the DrosC and D γ 2 antibodies did not immunoprecipitate Dgp71WD in the absence of γ -tubulin (Figure 4D). This result demonstrated that these antibodies against γ -tubulin did not nonspecifically bind to Dgp71WD. We found that when γ -tubulin and Dgp71WD were coexpressed, the two antibodies against γ -tubulin could immunoprecipitate both γ -tubulin and Dgp71WD (Figure 4D). On the basis of these results, we concluded that Dgp71WD and γ -tubulin directly interact with each other.

γ -Tubulin Interacts Directly with the Grip Motif-containing Subunits Dgrip128 and 163

We hoped to determine whether γ -tubulin interacted with Dgrips128 and 163, two grip motif-containing subunits thought to be part of the cap structure. γ -Tubulin was coexpressed with either Dgrip128 or Dgrip163. Antibodies

Figure 2. Dgp71WD is a γ TuRC subunit. (A and B) A peptide antibody raised against Dgp71WD immunoprecipitates the γ TuRC. Clarified *Drosophila* embryo extracts were immunoprecipitated with a control IgG from nonimmunized rabbits (NR) or specific antibodies against γ -tubulin (γ -tub) or Dgp71WD (71). The immunoprecipitates were separated by 10% SDS-PAGE and visualized by Coomassie Blue staining (A) or Western blotting (B), probing with antibodies against the γ TuRC subunits as indicated. Because the heavy chain of the rabbit antibodies used for the immunoprecipitations migrated with a similar molecular mass as γ -tubulin, we used mouse mAb (Sigma) against human γ -tubulin (that cross reacts with *Drosophila* γ -tubulin) to specifically detect γ -tubulin in B. (C) Dgp71WD comigrates with the γ TuRC on sucrose gradients. Clarified *Drosophila* embryo extract was sedimented on a 5–40% sucrose gradient in H100. The resulting fractions were separated by 10% SDS-PAGE and analyzed by Western blotting probing with peptide antibody against Dgp71WD. Antibodies against γ -tubulin and Dgrip163 were used to detect the γ TuRC at ~ 32 S (γ TuRC peak indicated with an asterisk). (D and E) Dgp71WD colocalizes with γ -tubulin on interphase (D) and mitotic (E) centrosomes in *Drosophila* embryos. 4, 6-Diamino-2-phenylindole (DAPI) was used to visualize DNA, and peptide antibody against Dgp71WD and the mAb against γ -tubulin (Sigma) were used to detect the respective proteins.

against each of the Dgrips and γ -tubulin were used to do reciprocal immunoprecipitations as described above. The immunoprecipitates were analyzed by SDS-PAGE followed by Coomassie Blue staining as well as by Western blotting. We found that the antibody against γ -tubulin immunoprecipitated γ -tubulin, Dgrip128, and Dgrip163, whereas the antibodies against Dgrip128 and Dgrip163 immunoprecipitated themselves and γ -tubulin (Figure 5, A and B). Therefore, γ -tubulin interacts directly with Dgrips128 and 163. Taken together, the above findings suggest that each of the

four grip motif-containing subunits interact directly with Dgp71WD and γ -tubulin.

Dgp71WD, Unlike the Grip Motif-containing γ TuRC Components, Does Not Facilitate γ -Tubulin Binding to GTP

We next hoped to develop an assay to compare the functional consequences of the binding of Dgp71WD to γ -tubulin with that of the grip motif-containing proteins. Because

Figure 3. Dgp71WD interacts with Dgrips84, 91, 128, and 163. Dgp71WD was coexpressed with Flag-tagged Dgrip84 (A), Flag-tagged Dgrip91 (B), untagged Dgrip128 (C), or Flag-tagged Dgrip163 (D) in Sf9 cells. Antibodies against Dgp71WD (A–D), Flag (A, B, and D), and Dgrips84 (A), 91 (B), 128 (C), and 163 (D) were used to immunoprecipitate the respective proteins from the cell lysates. The immunoprecipitated proteins were fractionated by SDS-PAGE followed by either Coomassie Blue staining (A–D, top panels) or Western blotting (A–D, bottom panels). Antibody indicates the heavy and light chains of the antibody molecules on the Coomassie Blue-stained gels. The heavy chain of the Flag mAb migrated slower than the heavy chains of the rabbit polyclonal antibodies. Asterisks indicate contaminating proteins present in the immunoprecipitations.

γ -tubulin in γ TuRC and γ TuSC can bind to guanine nucleotides (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000), we reasoned that the nucleotide binding of γ -tubulin could be

dependent on its interaction with other γ TuRC subunits. Therefore, we asked whether γ -tubulin expressed alone or together with Dgrip84 or Dgrip91 could bind to GTP. Using

Figure 4. Dgp71WD interacts with γ -tubulin. (A and B) Dgp71WD and γ -tubulin were coexpressed in Sf9 cells and then immunoprecipitated with antibodies against Dgp71WD (71), γ -tubulin (γ -tub), or nonimmunized rabbit IgG (NR). The immunoprecipitates were fractionated by 10% SDS-PAGE and then analyzed either by Coomassie Blue staining (A) or by Western blotting, probing with the antibody against Dgp71WD and the mAb against human γ -tubulin (Sigma). (C) Quantification of the expression levels of Dgp71WD and γ -tubulin. Dgp71WD and γ -tubulin were either expressed alone or together in Sf9 cells. The cell lysates were analyzed by Western blotting and probing with antibodies against Dgp71WD and γ -tubulin. (D) Two antibodies against γ -tubulin, DrosC, and D γ 2, the rabbit nonimmunized IgG (NR), and the antibody against Dgp71WD were used for immunoprecipitation of the lysates in C. The immunoprecipitates were analyzed by Western blotting and probing with antibodies against Dgp71WD and the mAb against human γ -tubulin.

Figure 5. γ -Tubulin interacts directly with Dgrips128 and 163. γ -Tubulin was coexpressed with either Dgrip128 or Dgrip163 in Sf9 cells and immunoprecipitated with control antibody (NR) or specific antibodies against γ -tubulin (γ -tub), Dgrip128 (128), or Dgrip163 (163). The immunoprecipitated proteins were analyzed by 10% SDS-PAGE followed by Coomassie Blue staining (A) or by Western blotting (B) probing with antibodies against the γ TuRC subunits. Nonspecific proteins that immunoprecipitate with all three antibodies are marked with an asterisk. Mouse mAb (Sigma) against human γ -tubulin was used to detect γ -tubulin in B.

the UV cross-linking assay developed previously (Oegema *et al.*, 1999), we found that γ -tubulin when expressed alone and purified, did not bind to GTP (Figure 6, A–C). However, when γ -tubulin was coexpressed with either Dgrip84 or Dgrip91 in Sf9 cells and purified, it was able to bind to GTP in the cross-linking assay (Figure 6, A–C). As expected, γ -tubulin in the baculovirus reconstituted γ TuSC also binds to GTP (Gunawardane *et al.*, 2000 and this study, Figure 6, A–C). These findings suggest that the interactions between the Dgrip84 and Dgrip91 with γ -tubulin facilitate γ -tubulin binding to GTP.

The above finding prompted us to ask whether Dgp71WD, Dgrip128, and Dgrip163 could also facilitate γ -tubulin to bind to GTP. γ -Tubulin was coexpressed with Dgp71WD, Dgrip128, or Dgrip163, isolated, and assayed for GTP binding using the UV cross-linking assay as above. The expression levels of Dgrip128 and Dgrip163 were about the same as that of Dgp71WD in these experiments (unpublished data). We found that γ -tubulin bound to GTP when coexpressed with either Dgrip128 or Dgrip163, but not when coexpressed with Dgp71WD (Figure 7, A–C). Furthermore, γ -tubulin could bind to GTP when coexpressed with all three subunits (Figure 7, A–C), suggesting that Dgp71WD did not inhibit GTP binding. These studies suggested that the interaction between γ -tubulin and the grip motif-containing subunits could facilitate γ -tubulin binding to GTP, but the interaction between γ -tubulin and Dgp71WD did not. From these studies, we conclude that the nature of the interaction of Dgp71WD with γ -tubulin differs from that of the grip motif-containing γ TuRC subunits.

DISCUSSION

To understand the function of γ TuRC, it is important to identify all of its subunits and study their interactions. We report here the identification of Dgp71WD, a new γ TuRC subunit that does not have grip motifs. We found that both Dgp71WD and γ -tubulin interacted directly with the subunits containing grip motifs. These findings shed new light on the structural organization of the γ TuRC.

Sequence analyses suggested that there are two additional *Drosophila* grip motif-containing proteins with predicted molecular masses of 79.8 kDa (AAF50536) and 223 kDa (AAF44968; Murphy *et al.*, 2001). The putative 79.8-kDa protein, which we will refer to as Dgrip79 hereafter, may correspond to one of the uncharacterized γ TuRC subunits in the ~75-kDa molecular mass range (Figure 1A). However, the putative 223-kDa protein may not be an integral subunit of the *Drosophila* γ TuRC, because all the *Drosophila* γ TuRC subunits are smaller than 223 kDa. If Dgrip79 is a γ TuRC subunit and if our estimation of a total of eight subunits in γ TuRC is accurate, we may have identified a complete set of *Drosophila* γ TuRC subunits.

Interestingly, six (Dgrips75, 79, 84, 91, 128, and 163) of eight γ TuRC subunits contain the conserved grip motifs. This sequence conservation suggests that the Dgrips interact with common proteins either within or outside of the γ TuRC. We showed that, consistent with this idea, Dgrips84, 91, 128, and 163 directly interacted with γ -tubulin and Dgp71WD, two γ TuRC subunits with no grip motifs (Gunawardane *et al.*, 2000 and this study). It will be important to determine whether the grip motifs in the Dgrips mediate these interactions.

The finding that all Dgrips interact with γ -tubulin directly is intriguing because it suggests that the Dgrips75, 79, 128, and 163 that were originally thought to be the cap subunits, directly contact γ -tubulin in the γ TuRC. If this is the case, the current γ TuRC model, which hypothesizes that the γ TuRC ring consists exclusively of γ TuSCs (Oegema *et al.*, 1999; Moritz *et al.*, 2000), would need to be revised. We speculate that each of the Dgrips75, 79, 128, and 163 could form dimers with γ -tubulin molecules. These dimers along with several γ TuSCs may be required to form the ring of the γ TuRC.

Structural studies revealed that WD repeats form a β -propeller fold that could mediate protein-protein interactions

Figure 6. γ -Tubulin binds GTP when coexpressed with Dgrip84 or Dgrip91. Flag-tagged γ -tubulin expressed alone or coexpressed with Dgrip84 and/or Dgrip91 was immunoprecipitated with Flag antibody-bound agarose beads. The immunoprecipitates were incubated with α - 32 P-GTP, UV cross-linked, and separated by 10% SDS-PAGE. The gel was first stained with Coomassie Blue to visualize the proteins (A), dried, and then subjected to autoradiography to detect the α - 32 P-GTP (B).

Asterisk in A indicates a contaminating protein present in Sf9 cells. The presence of γ -tubulin in the cross-linking reactions was further confirmed by Western blotting, probing with antibodies against γ -tubulin (C).

(Smith *et al.*, 1999). We suggest that Dgp71WD could provide a scaffold via its WD-repeats to tether all of the Dgrips together in the γ TuRC. Consistent with this idea, we found that four Dgrips (84, 91, 128, and 163) interact directly with Dgp71WD. Clearly, further structural and biochemical studies are necessary to determine whether some or all of the Dgrips75, 79, 128, and 163 participate in the formation of the γ TuRC ring. Also, it is important to determine whether and how any of these Dgrips participate in the formation of the cap structure of the γ TuRC.

We found that coexpressing any one of the grip motif-containing subunits, Dgrips84, 91, 128, and 163, with γ -tubulin was sufficient to promote γ -tubulin to bind to GTP. However, coexpressing γ -tubulin with Dgp71WD, which does not con-

tain grip motifs, did not facilitate γ -tubulin binding to GTP. This finding showed that the interactions between γ -tubulin and Dgrips have a significantly different consequence from the interaction between γ -tubulin and Dgp71WD. Furthermore, it suggests that the grip motif-containing subunits play a role in regulating the GTP binding properties of γ -tubulin. Because GTP is important for α - and β -tubulin function, we suspect that Dgrips may be important for γ -tubulin function. Consistent with this idea, we found that γ -tubulin expressed alone could not incorporate into γ TuRC in vitro; but coexpressing one of the Dgrips with γ -tubulin was sufficient for the incorporation (unpublished data).

Previously, human and *Chlamydomonas* γ -tubulins expressed alone using rabbit reticulocyte lysate and baculovi-

Figure 7. Dgp71WD does not facilitate the binding of γ -tubulin to GTP, whereas Dgrips128 and 163 do. Flag-tagged γ -tubulin was expressed alone or coexpressed with one or all three of the untagged Dgrip128, Dgrip163, and Dgp71WD and immunoprecipitated with Flag-antibody bound agarose beads. After washing with buffer containing no GTP, the immunoprecipitates were incubated with α - 32 P-GTP, UV cross-linked, and separated by 10% SDS-PAGE. The gel was stained by Coomassie Blue to visualize γ -tubulin (A), dried, and subjected to autoradiography to detect α - 32 P-GTP (B). (C) Western blotting of the immunoprecipitates confirmed the presence of γ -tubulin, Dgrips128, Dgrip163, and Dgp71WD in these experiments.

rus expression systems, respectively, were used to study the microtubule binding and nucleating activities of γ -tubulin (Li and Joshi, 1995; Vassilev *et al.*, 1995; Leguy *et al.*, 2000). However, whether the γ -tubulins could bind to guanine nucleotides was not determined (Li and Joshi, 1995; Vassilev *et al.*, 1995; Leguy *et al.*, 2000). Because we found that γ -tubulin expressed alone did not bind GTP, caution should be used in analyzing the function of γ -tubulin in the absence of Dgrips.

The interaction we observed between Dgp71WD and γ -tubulin should also be interpreted cautiously. Because γ -tubulin found in γ TuRC and γ TuSC can be cross-linked to GTP, the inability of γ -tubulin to bind to GTP when coexpressed with Dgp71WD may indicate that γ -tubulin does not assume the same structural conformation as in the γ TuRC. One possibility is that the γ -tubulin coexpressed with Dgp71WD was not folded properly. If so, the interaction between Dgp71WD and γ -tubulin that we observed may not reflect a true interaction in the γ TuRC. Alternatively, it is possible that the γ -tubulin expressed with Dgp71WD was folded correctly, but assumed a slightly different conformation to disfavor GTP binding in our *in vitro* assays. If so, the interaction between Dgp71WD and γ -tubulin is likely to reflect a true interaction in the γ TuRC. Structural studies of γ TuRC will be important to resolve whether γ -tubulin directly contact Dgp71WD in the complex.

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