Distinct Dgrip84 Isoforms Correlate with Distinct γ -Tubulins in *Drosophila*

Christiane Wiese

Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706

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γ -Tubulin is an indispensable component of the animal centrosome and is required for proper microtubule organization. Within the cell, γ -tubulin exists in a multiprotein complex containing between two (some yeasts) and six or more (metazoa) additional highly conserved proteins named gamma ring proteins (Grips) or gamma complex proteins (GCPs). γ -Tubulin containing complexes isolated from *Xenopus* eggs or *Drosophila* embryos appear ring-shaped and have therefore been named the γ -tubulin ring complex (γ TuRC). Curiously, many organisms (including humans) have two distinct γ -tubulin genes. In *Drosophila*, where the two γ -tubulin isotypes have been studied most extensively, the γ -tubulin genes are developmentally regulated: the “maternal” γ -tubulin isotype (named γ Tub37CD according to its location on the genetic map) is expressed in the ovary and is deposited in the egg, where it is thought to orchestrate the meiotic and early embryonic cleavages. The second γ -tubulin isotype (γ Tub23C) is ubiquitously expressed and persists in most of the cells of the adult fly. In those rare cases where both γ -tubulins coexist in the same cell, they show distinct subcellular distributions and cell-cycle-dependent changes: γ Tub37CD mainly localizes to the centrosome, where its levels vary only slightly with the cell cycle. In contrast, the level of γ Tub23C at the centrosome increases at the beginning of mitosis, and γ Tub23C also associates with spindle pole microtubules. Here, we show that γ Tub23C forms discrete complexes that closely resemble the complexes formed by γ Tub37CD. Surprisingly, however, γ Tub23C associates with a distinct, longer splice variant of Dgrip84. This may reflect a role for Dgrip84 in regulating the activity and/or the location of the γ -tubulin complexes formed with γ Tub37CD and γ Tub23C.

INTRODUCTION

The temporal and spatial control of microtubule nucleation and organization are essential for many fundamental cellular processes, including cell shape changes, intracellular transport, cell motility, partitioning of polarity signals during embryonic development, and, perhaps most importantly, the faithful segregation of chromosomes during cell division. In most animal cells, microtubules are nucleated and organized by the centrosome, which is composed of a pair of centrioles surrounded by the amorphous pericentriolar material (PCM) that appears to be the site of microtubule nucleation and anchoring (Gould and Borisy, 1977). Although efforts to generate an inventory of centrosomal components have made great progress in recent years (for example, Andersen *et al.*, 2003), the molecular organization of the centrosome itself is not well understood, and mechanistic descriptions of how centrosomal proteins contribute to microtubule formation and organization are yet to be obtained.

γ -Tubulin (Oakley and Oakley, 1989) is an indispensable component of the animal centrosome required for microtubule nucleation and organization in all eukaryotes (reviewed in Wiese and Zheng, 2006). Human γ -tubulin can

restore viability to *Schizosaccharomyces pombe* lacking endogenous γ -tubulin (Horio and Oakley, 1994), suggesting that γ -tubulins are functionally conserved in phylogenetically distant organisms. Within the cell, γ -tubulin exists in a multiprotein complex containing between two (in the case of *Saccharomyces cerevisiae*) and five or more (*S. pombe*, *Xenopus laevis*, *Drosophila melanogaster*, and humans) additional proteins named gamma ring proteins (Grips) or gamma complex proteins (GCPs). γ -Tubulin containing complexes isolated from *Xenopus* eggs or *Drosophila* embryos appear ring-shaped and have therefore been named γ -tubulin ring complex (γ TuRC; Zheng *et al.*, 1995). The non- γ -tubulin γ TuRC components are themselves highly conserved (Murphy *et al.*, 1998, 2001; Anders *et al.*, 2006; Tassin *et al.*, 1998; Martin *et al.*, 1998; Haren *et al.*, 2006; Lüders *et al.*, 2006). One of the major subcomplexes of the γ TuRC, which has been named the γ -tubulin small complex (γ TuSC; Oegema *et al.*, 1999), appears to be a tetramer composed of two molecules of γ -tubulin and one molecule each of two other Grips known as Spc97p and Spc98p in *S. cerevisiae*, Dgrip84 and Dgrip91 in *Drosophila melanogaster*, or GCP2 and GCP3 in mammals (reviewed in Wiese and Zheng, 2006). The tetramer was first described in *S. cerevisiae* (Knop and Schiebel, 1997), where it may be the only soluble γ -tubulin complex. The metazoan γ TuRC is thought to be composed of six or seven tetramers held together and capped by several additional proteins that copurify with γ -tubulin from *Xenopus* egg extracts or *Drosophila* embryos (Zheng *et al.*, 1995; Oegema *et al.*, 1999; Gunawardane *et al.*, 2003), but subcomplexes between γ -tubulin and non- γ TuSC Grips may also contribute to γ TuRC structure (Gunawardane *et al.*, 2000).

Consistent with a role for the γ TuRC in microtubule nucleation, γ -tubulin and its binding partners are localized to

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Address correspondence to: Christiane Wiese (wiese@biochem.wisc.edu).

Abbreviations used: γ tub, γ -tubulin; γ TuRC, γ -tubulin ring complex; γ TuSC, γ -tubulin small complex; MT, microtubule.

the PCM at the minus ends of the microtubules (Moritz *et al.*, 1995a,b; reviewed in Wiese and Zheng, 2006; Moritz and Agard, 2001). However, the molecular mechanism(s) for the function of the γ TuRC in microtubule nucleation are still unclear. In addition to facilitating nucleation of microtubules, γ -tubulin also seems to play a role in interphase microtubule organization (Jung *et al.*, 2001; Horio and Oakley, 2003), spindle assembly (Paluh *et al.*, 2000; Prigozhina *et al.*, 2001; Vogel and Snyder, 2000a,b; Sampaio *et al.*, 2001; Barbosa *et al.*, 2003; Lüders *et al.*, 2006), and spindle assembly checkpoint regulation (Vardy and Toda, 2000; Hendrickson *et al.*, 2001; Vardy *et al.*, 2002; Müller *et al.*, 2006; reviewed by Blagden and Glover, 2003). Little is known to date about how γ -tubulin or the Grips participate in each of these functions.

Curiously, the genomes of several organisms, including humans and flies, encode two distinct γ -tubulin genes (Zheng *et al.*, 1991; Wilson *et al.*, 1997; Tavosanis *et al.*, 1997; Wise *et al.*, 2000). In *Drosophila*, where the two γ -tubulin isotypes have been studied most extensively, the γ -tubulin genes are developmentally regulated: the "maternal" γ -tubulin isotype (named γ Tub37CD according to its location on the genetic map) is highly expressed in the ovary and is deposited in the egg, where it is thought to orchestrate the early embryonic cleavages (Wilson *et al.*, 1997; Llamazares *et al.*, 1999). Consistent with this idea, mutation of γ Tub37CD results in female sterility (Tavosanis *et al.*, 1997). In contrast, γ Tub23C is essentially ubiquitous and is required for viability, microtubule organization during somatic mitotic divisions, and male meiosis (Sunkel *et al.*, 1995; Sampaio *et al.*, 2001). Although both isotypes are present in early embryos, only γ Tub37CD is detected at centrosomes in syncytial embryos, whereas punctate structures containing γ Tub23C distribute throughout the cytoplasm (Wilson *et al.*, 1997). Later during development, both γ Tub23C and γ Tub37CD localize to centrosomes in cellularized embryos (Tavosanis *et al.*, 1997; Wilson *et al.*, 1997; Raynaud-Messina *et al.*, 2001). The extent to which each γ -tubulin isotype contributes to the assembly of female meiotic spindles remains to be resolved (Tavosanis *et al.*, 1997; Wilson and Borisy, 1998; Llamazares *et al.*, 1999).

Differences in the timing of their association with the centrosome have also been reported for the two γ -tubulin isotypes in *Drosophila* tissue culture cells (Raynaud-Messina *et al.*, 2001). In Kc23 cells, the centrosomal levels of γ Tub37CD have been reported to vary only slightly throughout the cell cycle, whereas centrosomal γ Tub23C levels increased sharply during late G2. Furthermore, γ Tub23C was also recruited to the microtubules of the mitotic spindle, whereas γ Tub37CD was never found on spindle microtubules. This suggests the existence of mechanisms that allow the cell to distinguish between the two γ -tubulin isotypes. Here, we set out to gain a better understanding of the biochemistry of γ Tub23C. We report that *Drosophila* γ Tub23C isolated from tissue culture cells forms bona fide ring complexes that include most of the same Grips as the γ TuRCs formed by γ Tub37CD. However, we were surprised to find that γ Tub23C and γ Tub37CD associate with distinct splice variants of Dgrip84/GCP2 that differ by 74 amino acids in their amino termini. We speculate that Dgrip84 may be involved in regulating the subcellular localization of γ -tubulin.

MATERIALS AND METHODS

Buffers and Reagents

The following buffers were used: HB: 50 mM K-HEPES, pH 7.6, 1 mM MgCl₂, 1 mM EGTA, 1 mM β -mercaptoethanol (β -ME) and protease inhibitor stock (1:200 final dilution; see below). H100: HB plus 100 mM NaCl; H200: HB plus

200 mM NaCl; and H500: HB plus 500 mM NaCl. Elution buffer (EB): H200 plus 100 μ M GTP and 1 mg/ml Dros23C or Dros37C peptide. Homogenization buffer (for embryos): H100 plus 10% glycerol, 1 mM PMSF and protease inhibitor stock (1:100 final dilution). Cell lysis buffer (for S2 and Kc cells): H200 plus 0.5% NP-40, 1 mM GTP, and 1 mM PMSF. Protease inhibitor stock: 10 mM benzamide-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. Sample buffer: 250 mM Tris, pH 6.8, 12% SDS wt/vol, 20% β -ME vol/vol, and 40% glycerol vol/vol. Tubulin was purified from the bovine brain and labeled with tetramethylrhodamine as described (<http://skye.med.harvard.edu>). For RNA work, aqueous solutions were treated with 0.05% diethyl pyrocarbonate (DEPC; Research Products International, Mount Prospect, IL) and autoclaved for 30 min.

Antibody Production

Dgrip84 antibodies were a kind gift from Y. Zheng (Carnegie Institution of Washington, Baltimore, MD) and are described in Oegema *et al.* (1999). Synthetic peptides (made by the Protein Synthesis facility of the University of Wisconsin, Madison Biotech Center) corresponding to the COOH-terminal 17 amino acids of γ Tub23C (Cys-PVDSKEDSRSVTSAGS; GenBank Accession no. P23257) or γ Tub37CD (Cys-QIDYQWSPAVEASKAG; GenBank Accession no. P42271) were used to raise and affinity purify rabbit polyclonal antibodies as described elsewhere (Field *et al.*, 1998). Peptides contained an additional N-terminal cysteine residue to facilitate coupling to KLH for antibody production, or to SulfoLink coupling gel (Pierce Biotechnology, Rockford, IL) for affinity purification. Specific antibodies were purified as described (Kellogg and Alberts, 1992), except that before affinity purification of the antisera with the immunogenic peptide, each serum was passed over a column corresponding to the nonimmunogenic γ -tubulin isotype. This procedure depleted potential cross-reacting antibodies and thus ascertained the specificity of the affinity-purified antibodies.

Drosophila S2 Cell Tissue Culture

Schneider S2 cells (Schneider, 1972) were cultured in Schneider's *Drosophila* medium as described by the supplier (Invitrogen, Carlsbad, CA). Cells to be used for extract preparation were grown for 4 d at 27°C in ~70 ml *Drosophila* medium in T-150 flasks. Cells were collected by centrifugation and washed twice with PBS, the volume of the cell pellet was estimated, and the cells were stored at -80°C until use.

PEG Precipitation

PEG (polyethylene glycol P-2139; average mol wt 5–8000; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1–5% (from a 30% stock in H100) to clarified *Drosophila* embryo or S2 cell extract. The mixture was incubated on ice for 20 min, spun at 17,000 rpm for 10 min in a JA20 rotor (Beckman, Fullerton, CA), and the supernatant was removed. The pellets were resuspended in a volume corresponding to the original extract volume of H200 plus 0.05% NP-40 and 100 μ M GTP and were clarified at 17,000 rpm for 10 min in a JA20 rotor. Equivalent portions of supernatant and pellet fractions were then analyzed by sucrose gradient fractionation, SDS-PAGE, and Western blotting.

Immunoprecipitation of γ -Tubulin-containing Complexes from *Drosophila* Embryo Extract

Drosophila embryo extract was prepared by homogenizing *Drosophila* embryos in homogenization buffer as described (Moritz *et al.*, 1998; Oegema *et al.*, 1999). Embryo extract was frozen in aliquots in liquid nitrogen, stored at -80°C, and used within 2 wk of preparation. Clarified extract was prepared by centrifugation of thawed crude extract for 1 h at 50,000 rpm in a rotor (SW55; Beckman) at 4°C. γ -Tubulin complexes were immunoprecipitated from the supernatant essentially as described (Oegema *et al.*, 1999), except that the PEG precipitation step was omitted. Briefly, anti- γ -tubulin antibody (23C or 37CD; ~8 μ g/ml) was incubated with the supernatant at 4°C for 1 h with gentle rotation. Per 12 ml of clarified extract, 35 μ l of protein A affiprep beads (Bio-Rad Laboratories, Hercules, CA) were equilibrated in wash buffer (H200 plus 0.05% NP-40, 100 μ M GTP, and 1 mM PMSF) and added to the antibody-extract mix. After a further incubation for 1 h with gentle rotation, the beads were collected and washed six times with wash buffer and were incubated for 16–18 h at 4°C with an equal volume of EB containing the respective peptide. γ -Tubulin complexes were collected by loading an additional 40 μ l of EB onto the beads and collecting the supernatant.

Immunoprecipitation of γ -Tubulin-containing Complexes from *Drosophila* Tissue Culture Cells

Drosophila tissue culture cell extract was prepared by resuspending thawed S2 cell pellets in 3 vol of cell lysis buffer, brief vortexing, and incubation on ice for 10 min. Cell debris was removed by a 10-min spin (at 4°C) at 2400 rpm in a Beckman table-top centrifuge. The supernatant was then incubated with

antibody and processed as described above for the immunoisolation of γ -tubulin complexes from embryo extract.

Sucrose Gradient Sedimentation

Sucrose gradients (5–40%) were poured and run as described by Oegema *et al.* (1999). Briefly, gradients were poured by hand as step gradients (five steps of equal volume) in H100 plus 1 mM GTP and allowed to diffuse into continuous gradients. After centrifugation at 50,000 rpm in a Beckman TLS-55 rotor for 4.5 h at 4°C, gradients were fractionated by hand (16 fractions plus pellet). Fractions were separated on a 10% SDS-PAGE gel and analyzed by Western blot or Coomassie stain, as indicated. For some experiments, each fraction was precipitated by addition of 10% trichloroacetic acid (TCA) and centrifugation (30 min) in an Eppendorf centrifuge after a 30-min incubation on ice. Pellets were resuspended in SDS-sample buffer and analyzed by SDS-PAGE and Western blot.

Electron Microscopy

Negative staining electron microscopy of peptide-eluted complexes was described elsewhere (Zheng *et al.*, 1995; Wiese and Zheng, 2000). Samples were viewed at 80 kV in a Philips Tecnai 12 microscope (Mahwah, NJ) equipped with a cooled CCD camera.

RT-PCR to Detect Dgrip84/GCP2 Isoforms

Total RNA was extracted from 100 μ l of frozen 0–4 h *Drosophila* embryos or $\sim 10^7$ S2 cells by homogenizing the cells in Trizol reagent (Invitrogen) and extracting the RNA according to the manufacturer's recommendations.

The following primers were used to reverse transcribe Dgrip84 mRNA (all isoforms) and amplify specific regions of the gene to distinguish the isoforms: the first strand cDNA template was synthesized from the extracted RNA using the ImPromII-Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's recommendations and using the Dgrip84/GCP2 gene-specific primer, 5'-CTCTACACACATCTTGTC-3'. Isoform-specific PCR primers used to differentiate the Dgrip84 isoforms were as follows: To distinguish the 5' end of the "A"-isoform from B/C using three PCR primers: forward primer 5'-AAAGAGCTCAATTGCC-3' (specific for the 5' untranslated region of isoform A) combined with reverse primer 5'-TCGAG-CAGCATACTC-3' (amplifies all isoforms) gives an 885-base pair band if isoform A is present, no product for isoform B or C; forward primer 5'-CAGTCGATCTAGCCGTTG-3' (specific for isoforms B and C, because this primer targets a region that is spliced out in isoform A) with reverse primer 5'-TCGAGCAGCATACTC-3' (amplifies all isoforms) gives an 855-base pair band if isoform B or C is present. To test for the presence of the 3' insertion that distinguishes isoform C from isoforms A and B, we used forward primer 5'-GCTAGTCTCTATCGAG-3' and reverse primer 5'-CTGTGATCTGCTT-GAGG-3'. This primer pair amplifies a 500-base pair band if the insert is present, or a 400-base pair band if the insert is absent. PCR products were cloned and sequenced to confirm their identity.

RESULTS

To analyze γ Tub23C and its interacting proteins we began by raising antibodies specific for γ Tub23C or γ Tub37CD (Figure 1). Sequence alignments and molecular modeling (Figure 1, A and B) revealed that the differences between γ Tub23C and γ Tub37CD (a total of 81 amino acids) did not cluster to defined regions within the γ -tubulin molecule but were distributed across most of the surfaces (Figures 1B and Supplementary Figure S1). The biggest difference between γ Tub23C and γ Tub37CD was a 14-amino acid insertion near the C-terminus of γ Tub23C. We took advantage of this difference to generate γ -tubulin isotype-specific antibodies by immunizing rabbits with synthetic peptides corresponding to the C-terminal 17 amino acids of each γ -tubulin isotype (see *Materials and Methods*). Dot-blot analysis confirmed that the affinity-purified antibodies were specific for their respective immunogenic peptides and did not cross-react with the nonimmunogenic peptide (Figure 1D). As expected, the antibodies recognized bands of ~ 50 kDa (γ Tub37CD antibodies) and ~ 52 kDa (γ Tub23C antibodies), respectively, on Western blots of embryo extracts (Figure 1E) or of purified γ -tubulin complexes (Figure 1F). In addition, the γ Tub23C antibodies recognized a band of ~ 50 kDa in S2 (*Drosophila* tissue culture) cell extracts, whereas no band corresponding to γ -tubulin was recognized by the γ Tub37CD antibodies in

S2 cell extracts (Figure 1E). This suggests that our S2 cell line does not express γ Tub37CD, or only undetectably low levels.

Dgrip84 Migrates Differently in *Drosophila* Embryos and Tissue Culture Cells

We next wanted to understand whether γ Tub23C formed γ TuRC-like complexes. For this, we first asked whether the Dgrips that interact with γ Tub37CD in early *Drosophila* embryos also exist in cells that only express γ Tub23C. Extracts of *Drosophila* tissue culture cells (Schneider S2 or Kc) or embryos were Western blotted and probed for individual γ TuRC proteins (Figure 2A). This analysis revealed that Dgrip163/GCP6, Dgrip128/GCP5, Dgrip98/GCP3, and γ Tub23C migrated similarly in extracts made from embryos or tissue culture cells. In contrast, Dgrip84/GCP2 behaved differently in embryos and tissue culture cells: no band corresponding to the embryonic Dgrip84 was found in the tissue culture cell lines. Instead, two novel bands appeared that migrated at ~ 72 and ~ 100 kDa (Figure 2Af). To determine which, if either, of the novel bands might be a subunit of the γ TuRC, we analyzed extracts of S2 (Figures 2B and 3B) or Kc (Figure 3C) cells on 5–40% sucrose gradients and probed Western blots of gradient fractions for various γ TuRC subunits. This analysis showed that the ~ 72 -kDa band migrated near the top of the sucrose gradient and thus appeared to be unrelated to the γ TuRC or the γ TuSC. Whether or how the ~ 72 -kDa band is related to Dgrip84 is not yet clear. In contrast, the 100-kDa band migrated similar to γ Tub23C in tissue culture cells (Figure 2B) and like Dgrip84 in embryos (Figure 3D). Thus, our results strongly suggested that the ~ 100 -kDa band may be the Dgrip84 of somatic cells.

Two possibilities exist to explain the increased size of Dgrip84 in tissue culture cells: the protein might be differentially modified by secondary modifications, or different splice variants of the protein might be expressed. Database analysis revealed the existence of three potential splice variants of Dgrip84 (diagramed in Figure 4). The shortest isoform, Dgrip84-C (Accession no. NP_523409), differed from the longest isoform, Dgrip84-A (Accession no. NP_728264), in two ways: a 33-aa insertion near the C-terminus in Dgrip84-A, and a 74-aa extension at its N-terminus. Isoform Dgrip84-B (Accession no. NP_728265) was intermediate in that it had the C-terminal insertion but lacked the N-terminal extension. A fourth configuration, lacking the C-terminal insertion but having the N-terminal extension, was also possible but was not reported in the database. We named this configuration Dgrip84-D (Figure 4, A–C).

To gain a better understanding of which of the Dgrip84 isoforms was expressed in embryos and tissue culture cells, we examined Dgrip84 mRNA and protein during early fly development. For this, we collected staged embryos (4-h time intervals for 24 h) or unsynchronized S2 cells and made extracts for Western blot analysis (Figure 4D). Alternatively, we used the extracts to isolate total mRNA for Northern blot analysis (Figure 4E) or RT-PCR (Supplementary Figure S2).

Western blots of staged embryos probed with anti-Dgrip84 antibodies showed that Dgrip84 (marked "D" in Figure 4D) migrated more slowly in older embryos compared with younger ones. The slower-migrating form of Dgrip84 was detectable within 8–12 h of development, and its relative levels increased as the levels of the shorter isoform (marked "C" in Figure 4D) slowly declined.

To determine whether the appearance of the slower-migrating Dgrip84 protein could be explained by a switch to a longer Dgrip84 isoform, we examined the isoforms expressed in staged embryos by Northern blot analysis. North-

Figure 1. Analysis of *Drosophila* γ Tub23C and γ Tub37CD and isotype-specific antibodies. (A) Sequence alignment between γ Tub37CD and γ Tub23C reveals that nonconserved differences between the two isotypes are scattered throughout the primary sequence. Light gray, identities; medium gray, conserved changes; red, differences that change the character of the amino acid; blue, amino acid insertions in γ Tub23C relative to γ Tub37CD; the transparent amino acids near the C-termini of the molecules are not represented in the structural model. (B) A hypothetical structural surface model of γ Tub23C (generated by the structure prediction program SwissModel; Peitsch, 1995; Guex and Peitsch, 1997; Schwede *et al.*, 2003) is based on the crystal structure for human γ -tubulin (Aldaz *et al.*, 2005). To highlight the residues not conserved between the isotypes, the backbone of the residues whose character is significantly different between γ Tub23C and γ Tub37CD are colored in red and the M-loop insertion in γ Tub37CD that is not present in γ Tub23C is colored in blue, as in A. Snapshot images of different faces of the molecule are shown in each panel, as indicated in the upper left-hand corner. The orientation of the snapshots are based on the observation that γ -tubulin can roughly be modeled as a cube, and on the orientation of α and β tubulin within the microtubule lattice, as illustrated in (C). The snapshots illustrate two important points: first, the differences between the two γ -tubulins are not randomly distributed throughout the molecule but map mainly to the surfaces, presumably leaving the core of the structure largely unaffected (see also

Figure 2. *Drosophila* tissue culture cells express a slower migrating version of Dgrip84. (A) Western blot analysis of γ TuRC components in embryos, S2 cells, and Kc cells, as indicated above the lanes. Probes are indicated below the blots and are as follows: (a) γ Tub37CD, (b) γ Tub23C, (c) Dgrip98/GCP3, (d) Dgrip128/GCP5, (e) Dgrip163/GCP6, and (f) Dgrip84/GCP2. The embryonic "cognate" proteins are indicated by arrowheads on the left of each panel. Asterisks in f denote two bands (~100 and ~75 kDa) in tissue culture cells that are recognized by the Dgrip84 antibody. Note the absence in the tissue culture cells of a band corresponding to γ Tub37CD in panel a, and the absence of a band corresponding to embryonic Dgrip84 in panel f. (B) Sucrose gradient analysis of S2 cell extracts reveals that the slower migrating protein recognized by the Dgrip84 antibodies migrates similarly to γ Tub23C. Sucrose gradient fractions were probed for γ TuSC components as indicated on the right. The positions of γ TuSC and γ TuRC are indicated below the blot. Asterisks on the left denote the two proteins in tissue culture cell extracts that are recognized by the Dgrip84 antibody. Only the ~100-kDa protein migrates like the embryonic Dgrip84 (compare with Figure 3).

Figure 1 (cont). Supplementary Figure S1). Second, the differences map to all six faces of the cube. Corresponding ribbon models can be found in the online supplementary material, Figure S1. (D) Dot-blot analysis of affinity-purified antibodies. Aqueous solutions of each peptide were spotted onto a piece of nitrocellulose membrane, and the membrane was probed with each antibody, as indicated. The antibodies do not cross-react with each other. (E) Western blot of total extracts from embryos (emb, lanes 1 and 3) or S2 cells (S2 cells, lanes 2 and 4) probed with antibodies to γ Tub37CD (lanes 1 and 2) or γ Tub23C (lanes 3 and 4). Positions of molecular weight markers are indicated on the right. (F) Western blot of purified γ -tubulin complexes. Lane 1, γ Tub23C complexes probed with γ Tub37CD antibodies; lane 2, γ Tub37CD complexes probed with γ Tub23C antibodies. The ~40-kDa band recognized by the γ Tub37C antibodies in embryo extracts probably shares an epitope with γ Tub37CD, as this band cross-reacts with antisera produced by several different rabbits (our unpublished observation); however, this protein apparently does not coimmunoprecipitate with the γ TuRC and thus did not interfere with the analysis of the purified complex.

Figure 3. Sucrose gradient analysis of γ -tubulins and Dgrips in embryos and tissue culture cells. (A) Embryo extracts, 0–8 h, were analyzed on 5–40% sucrose gradients. Gradient fractions were probed for Dgrips and γ -tubulins as indicated on the right. (B and C) Extracts made from Kc cells (B) or S2 cells (C) were analyzed on sucrose gradients as described for A. D. Individual panels from A to C were regrouped to allow a more direct comparison for each γ TuRC component between the migration patterns in embryos and tissue culture cells. γ Tub23C migrates differently in embryos, but in S2 and Kc cells resembles the behavior of γ Tub37CD. For Dgrip84, only the ~100-kDa bands are shown in S2 and Kc cells.

ern blots showed that the shorter isoform of Dgrip84 expressed in early embryos (0–4 h) was replaced by a longer isoform later in development that was also present in S2 cells (Figure 4E). To identify the specific isoforms present in early embryos and S2 cells, we first designed a strategy to identify the isoform by RT-PCR using specific primers (diagrammed in Supplementary Figure S2). Our results were then confirmed by direct sequencing of the RT-PCR product. Consistent with results from previous work (Oegema *et al.*, 1999; Gunawardane *et al.*, 2000), we found that the isoform present in early embryos lacked both the N-terminal extension and the C-terminal insertion. This suggested that the embryos expressed mainly the "C"-isoform of Dgrip84. S2 cells, on the other hand, expressed mRNAs that contained

Figure 4. Dgrip84 isoforms in *Drosophila*. (A) Diagram of the relationships between different Dgrip84 isoforms. The position of the 33-amino acid C-terminal insertion and the 74-amino acid N-terminal extension are indicated. Theoretical molecular weights, number of amino acids, and pI are indicated on the right for each isoform (A–D). Isoforms A–C are currently present in the database (Accession no. NP_728264, NP_728265, and NP_523409, respectively). The isoform found in S2 cells (see below) has the extension but not the insertion. We named this the “D” isoform. (B) Amino acid sequence of Dgrip84 isoform A. The N-terminal extension and C-terminal insertion are indicated by gray shading and a box, respectively. (C) Gene structure of the Dgrip84 isoforms. Numbers below the diagram indicate the number of nucleotides in each exon (top row) or intron (bottom row). (D) Western blot of staged embryos. *Drosophila* embryos were collected for 4 h and were then aged for the times indicated above the blots. Maximum age was 24 h. Embryos were then homogenized, loaded onto SDS-PAGE gels, and Western blotted. The blots were probed with antibodies to Dgrip163, γ Tub37CD, γ Tub23C, or Dgrip84, as indicated on the right. The positions of the slower migrating (“C”) and faster-migrating (“D”) Dgrip84 bands are indicated on the left. (E) Northern blot of staged embryos probed with a Dgrip84 probe common to all isoforms. Embryos were collected and aged as in D. Early embryos (0–4 h) express a shorter mRNA than later embryos or S2 cells. To determine the identity of the isoforms, we performed RT-PCR and sequenced the relevant regions of cDNAs recovered from early embryos or S2 cells (see Supplementary Figure S3). This analysis shows that early embryos express the “C” isoform, whereas S2 cells express the “D” isoform. The reduction in signal for the older embryos (starting with 12–16-h embryos) may be due to sample degradation, although our loading control (rp49; DeZazzo *et al.*, 2000) remained unchanged.

the N-terminal extension. However, we were surprised to find no evidence for the C-terminal insertion. This suggested that the major isoform of Dgrip84 expressed in S2 cells is the previously unknown, novel “D” splice variant of Dgrip84 (Figure 4A). Together, these results suggested that the expression of Dgrip84 splice variants is developmentally regulated.

To confirm that the longer Dgrip84 splice variant is indeed a component of the γ -tubulin complex, we used our γ -tubulin isoform-specific or Dgrip84 antibodies to immunopre-

cipitate protein complexes from embryos or S2 cells and compared these by Coomassie-stained SDS-PAGE gels and Western blot (Figure 5). With the exception of the band that corresponds to Dgrip84, similar sets of proteins were precipitated from S2 tissue culture cells and from 0- to 3-h embryos using antibodies directed against Dgrip84 (Figure 5Aa), and their protein profile closely resembled the γ Tub37CD and γ Tub23C complexes isolated from embryos and S2 cells, respectively (Figure 5Ab; Oegema *et al.*, 1999). This suggested that both Dgrip84 splice variants are compo-

Figure 5. Dgrip84-D is a component of the γ TuRC. (A) Comparison between protein complexes immunoprecipitated with various antibodies. Panel a, Coomassie-stained gel of proteins that coimmunoprecipitate with Dgrip84 antibodies from embryo (lane 2) or S2 cell (lane 3) extracts. γ TuRC proteins are highlighted by arrowheads on the right, positions of molecular-weight markers are indicated on the left. Panel b, γ -tubulin complexes isolated from S2 cells using antibodies to γ Tub23C (lane 1), or from 0- to 8-h embryos using antibodies to γ Tub37CD (lane 2). Complexes isolated from S2 cells with antibodies to Dgrip84 or γ Tub23C resemble each other, as do complexes isolated from embryos by antibodies to Dgrip84 or γ Tub37CD. Complexes isolated from S2 cells lack a band corresponding to the embryonic Dgrip84; however, a new band appears above Dgrip91. (B) Negative-staining electron microscopy of γ Tub23C complexes isolated from S2 cells. Arrowheads in the left panel point to individual ring complexes. Scale bars, 25 nm in the left panel, 100 nm in the right panel. (C) Western blot of proteins that copurify with γ Tub37CD from early (0–8 h) embryos (lane 1), with γ Tub23C from late (8–16 h) embryos (lane 2), or with γ Tub23C from S2 cells (lane 3). Antibodies to Dgrips used to probe the Western blot are indicated on the right. The asterisks mark the two Dgrip84 isoforms that copurify with γ Tub37CD or γ Tub23C. The multiple bands in lane 3 might represent additional Dgrip84 isoforms or degradation products.

nents of γ -tubulin complexes. One important difference between S2 and embryonic γ -tubulin complexes was the absence of the band corresponding to the embryonic Dgrip84 and a concomitant appearance of a $\sim$ 100-kDa band in the S2 γ -tubulin complexes. Thus, consistent with the findings described above, the γ -tubulin complexes isolated from embryos or S2 cells differed by the associated Dgrip84 splice variant.

Next, we wanted to understand the structure of the S2 cell γ -tubulin complexes in greater detail. Using the γ Tub23C antibody, we adapted the strategy for isolating γ Tub37CD complexes from early embryo extracts (Oegema *et al.*, 1999) to isolate γ -tubulin complexes from S2 cell extracts (Figure 5Ab). In this strategy, γ -tubulin complexes are immunoprecipitated using the affinity-purified peptide antibodies, and the complexes are then eluted from the affinity beads with the specific synthetic peptide against which the antibodies were raised. This allowed us to examine the complexes by negative staining electron microscopy (Figure 5B), which showed that the purified γ Tub23C complexes appeared as $\sim$ 25-nm rings that closely resembled the embryonic γ TuRC_{37CD} (Zheng *et al.*, 1995; Oegema *et al.*, 1999). We therefore concluded that γ Tub23C formed a “canonical” γ TuRC in S2 cells.

The purified complexes were further analyzed by Western blotting with antibodies directed against embryonic Dgrips (Figure 5C). The Dgrips we tested (163/GCP6, 128/GCP5, and 98/GCP3) all copurified with γ Tub23C complexes isolated from late embryos (8–16 h) or S2 cells and migrated similarly on a 10% SDS-PAGE gel as their early embryonic counterparts (Figure 5C). Consistent with the results described above, the γ -tubulin complexes contained distinct but immunologically related Dgrip84 isoform(s), depending on which γ -tubulin isotype was present: γ Tub37CD correlated with a faster migrating Dgrip84, whereas the presence of γ Tub23C correlated with a slower migrating isoform. Interestingly, a small amount of γ Tub23C copurified with γ Tub37CD and vice versa (Figure 5C; see also Figures 1F and 6C), suggesting that some γ TuRCs may contain both γ -tubulin isotypes.

Our analysis also revealed important differences between γ Tub23C present in early embryos and γ Tub23C present in late embryos or S2 cells (Figures 5 and 6). Although the γ Tub23C antibodies immunoprecipitated a γ TuRC-like complex from late embryos (Figure 5C) or S2 cells (Figure 5, Ab and C), they failed to precipitate γ Tub23C, or a γ TuRC-like complex, from early embryos (Figure 6A). To understand whether γ Tub23C exists in a γ TuRC-like complex in early embryos, we examined embryo extracts (0–8 h) by sucrose gradient analysis and Western blotting (Figure 6B). Under the conditions used (100 mM salt, 5–40% sucrose; 16 fractions), γ Tub37CD distributed mainly in two peaks that corresponded to the previously characterized γ TuRC (fractions 13–15) and γ TuSC (fractions 6–8; Oegema *et al.*, 1999). γ Tub23C showed a distinct (albeit overlapping) distribution: only a small portion of γ Tub23C was found in the same fractions as the γ TuRC_{37CD}. Instead, γ Tub23C peaked in fractions 11 and 12 and was distributed broadly in fractions 4–9 without forming a distinct peak (Figure 6B). Surprisingly, the “large” γ Tub23C complex (fractions 11 and 12) was noticeably smaller than the γ TuRC_{37CD}. Similarly, the broad distribution of γ Tub23C in the lower fractions (4–9) suggested that γ Tub23C might form a smaller complex, perhaps resembling the γ TuSC, but this complex may be distinct from and/or more heterogeneous than the γ TuSC_{37CD}. We concluded that γ Tub23C migrated as a large complex in early embryos, but that this complex is mostly distinct from

Figure 6. γ Tub23C migrates as a large complex in early embryos that is distinct from the embryonic γ TuRC. (A) Coomassie gel of proteins that precipitate from early embryos with antibodies to γ Tub23C (lane 2) or γ Tub37CD (lane 3). Positions of γ TuRC components are indicated on the right. Lane 1, molecular-weight markers (sizes of markers are indicated on the left). (B) Sucrose gradient analysis of extracts made from 0- to 8-h *Drosophila* embryos. Western blot of 0-8 h embryo extracts fractionated on a sucrose gradient probed with antibodies specific for each γ -tubulin isotype or Dgrips 163, 128, 98, and 84, as indicated on the right. The positions of the γ TuRC (fractions 13-15), the γ TuSC (fractions 6-8), and the peak fractions for γ Tub23C (fractions 11 and 12) are indicated below the blot. The gel is loaded such that the lighter sucrose fractions (starting at 5%) are on the left, and the heavier fractions (up to 40%) are on the right. Fraction numbers are indicated above the blots. Load, 1 μ l of starting material was loaded as a control. (C) γ Tub23C and γ Tub37CD can be distinguished by their sensitivity to polyethylene glycol (PEG). PEG (2.5%) was added to extracts, and precipitated proteins were separated from soluble proteins by centrifugation. Supernatant (S) and resuspended pellet (P) fractions were then fractionated on sucrose gradients, run on a SDS-PAGE gel, and blotted for γ Tub23C or γ Tub37CD as indicated on the right.

the γ TuRC (Figure 6, A and B). These conclusions were further supported by experiments using differential precipitation of extract proteins with increasing concentrations of polyethylene glycol (PEG). γ Tub37CD and its associated proteins precipitated nearly quantitatively in the presence of 2.5% PEG, whereas a significant portion of γ Tub23C remained in the supernatant under these conditions. Sucrose gradient analysis of the supernatant and pellet fractions from the 2.5% PEG precipitation showed that the γ Tub37CD and γ Tub23C complexes could be separated from one an-

other under these conditions (Figure 6C). Thus, we concluded that although both γ -tubulin isotypes are present in complexes in early embryos, γ Tub23C complexes are mostly distinct from the γ TuRC_{37CD}.

In contrast, γ Tub23C comigrated with the Dgrips in tissue culture cells (Figure 3; see also Figure 2B), consistent with the observation that it forms a γ TuRC in these cells (see above). Although it is not yet clear at which developmental stage γ Tub23C switches from the "embryonic" complex to become part of the γ TuRC, or how this process is regulated, these findings support the idea that the formation of γ TuRCs containing γ Tub23C is developmentally controlled.

DISCUSSION

Distinct patterns of recruitment to the centrosome and spindle microtubules exhibited by γ Tub23C and γ Tub37CD in tissue culture cells and syncytial embryos (Sunkel *et al.*, 1995; Wilson *et al.*, 1997; Raynaud-Messina *et al.*, 2001, 2004) prompted us to examine the molecular compositions of the complexes formed by each of the two *Drosophila* γ -tubulin isotypes. We reasoned that differences in their localization might be regulated by intrinsic differences between the γ -tubulin isotypes or their binding partners, or both.

We analyzed γ -tubulin complexes in two different systems: in *Drosophila* embryos, where γ Tub37CD is the major γ -tubulin isotype, and in Schneider S2 tissue culture cells, where γ Tub23C is the major γ -tubulin isotype and γ Tub37C plays only a minor role, if any (Raynaud-Messina *et al.*, 2004). Based on the observation that γ -tubulin and its interacting proteins are highly conserved among distantly related species, it was reasonable to suspect that γ Tub23C forms complexes in *Drosophila* cells that closely resemble the γ TuRC_{37CD}. Here, we provide the first experimental evidence that this is indeed the case. We show that previously identified γ -tubulin-interacting proteins (Dgrips) copurify with γ Tub23C isolated from tissue culture cells, and together these proteins form a ring-shaped complex that closely resembles the γ TuRC_{37CD} when viewed by negative staining electron microscopy. Our studies revealed two unexpected results: 1) γ Tub23C forms a large complex in early embryos that is distinct from the γ TuRC, and 2) expression of different Dgrip84 isoforms is developmentally regulated and correlates with expression of different γ -tubulin isoforms. These findings support the notion that the two *Drosophila* γ -tubulins interact with different binding partners and perhaps interact differently with similar binding partners.

What Is the Role of the Embryonic γ Tub23C Complex?

In embryos, γ Tub23C distributes in punctate patterns not associated with centrosomes, whereas γ Tub37CD localizes to centrosomes and spindle poles during early embryonic divisions (Wilson *et al.*, 1997). It has been postulated that only γ Tub37CD is utilized at centrosomes during embryonic cleavages (Wilson *et al.*, 1997). Consistent with this, female meiosis and nuclear divisions during early embryogenesis specifically require γ Tub37CD, and γ Tub37CD mutants are female sterile despite the presence of γ Tub23C in the embryos (Tavosanis *et al.*, 1997; Tavosanis and Gonzales, 2003). This suggests that the γ -tubulin isotypes are not functionally redundant during embryogenesis. Our results provide a potential biochemical explanation for these observations. We propose that the embryonic γ Tub23C complex may represent a type of 'storage form' of γ Tub23C. This is suggested by the observation that γ Tub23C could not be immunoprecipitated from early embryos, indicating that the epitope recognized by the antibody was not accessible. We speculate

that the storage particle might include molecular chaperones of the TriC protein family, which have been shown to interact with and aid in the folding of γ -tubulin (Melki *et al.*, 1993). We found that the large γ Tub23C complexes persisted for at least the first 8 h of development, suggesting that the availability of γ Tub23C is very tightly regulated during early fly development. Full analysis of the embryonic γ Tub23C complex awaits methods to purify the complex that do not rely on antibody affinity chromatography. How the association of γ Tub23C with the putative storage particle is regulated and how γ Tub37CD escapes recruitment to the particle remains a mystery.

What Is the Importance of the Different Isoforms of Dgrip84/GCP2 in *Drosophila*?

To date, *Drosophila* Dgrip84 is the only member of the GCP2 protein family that has been reported to exist as more than one splice variant. Database analysis reveals that at least two *Drosophila* species possess the 74-amino acid N-terminal extension, *D. melanogaster* and *D. pseudoobscura*. It is possible that unique structural features of one or the other Dgrip84 isoform facilitate the particularly rapid centrosome assembly of the early *Drosophila* embryo, as has previously been postulated for γ Tub37CD (Wilson *et al.*, 1997). Consistent with this hypothesis, Dgrip84 has recently been implicated in centrosome assembly and separation (Colombié *et al.*, 2006). GCP2 (and GCP3) family members have also been implicated in γ TuRC recruitment to the spindle pole body in *S. cerevisiae* and to the centrosome in metazoa (Knop and Schiebel, 1997, 1998; Nguyen *et al.*, 1998; Barbosa *et al.*, 2000; Vinh *et al.*, 2002; Takahashi *et al.*, 2002; Zimmerman *et al.*, 2004; Kawaguchi and Zheng, 2004; Vérollet *et al.*, 2006). The precise role of GCP2 family members in γ TuRC function and recruitment remains to be elucidated.

Our analysis of Dgrip84 isoforms showed that the isoform expressed in embryos corresponds to the shortest splice variant, whereas the isoform expressed in older embryos and S2 cells corresponds to a previously unreported variant of Dgrip84 that has an N-terminal extension but lacks the reported C-terminal insertion. To date, we have been unable to find experimental evidence for the existence of Dgrip84 isoforms that possess the C-terminal insertion (isoforms A and B). It is possible that these isoforms are expressed only at very low levels or that they are expressed only in certain tissues. The roles of the extension or the insertion in Dgrip84 function are not yet clear, but we noted that the developmental switch to the longer isoform of Dgrip84 correlated with the appearance of γ TuRCs composed of γ Tub23C. Although thus far this is merely a correlation, it is tempting to speculate that the N-terminal Dgrip84 extension aids in folding or assembly of the somatic γ TuRC, or is required for incorporation of γ Tub23C into the γ TuRC. Experiments to address these questions are currently underway.

Chimeric γ TuRCs to Fine-Tune Microtubule Organization?

The metazoan γ TuRC contains 12–15 γ -tubulin molecules (Zheng *et al.*, 1995; reviewed in Jeng and Stearns, 1999; Wiese and Zheng, 2006), most of which are thought to be arranged in tetrameric subcomplexes (γ TuSCs) that contain two copies of γ -tubulin. Having two γ -tubulin isotypes in the same cell prompts a number of important questions: can both γ -tubulin isotypes coexist in the same γ TuSC? Can both isotypes coexist in the same γ TuRC? How does the cell distinguish between the γ -tubulin isotypes? Do the γ -tubulin isotypes perform different functions? How does the cell transition from using primarily one isotype to using the

other, and how does the activity of a chimeric γ TuRC (for which we found evidence by both sucrose gradient analysis and analysis of purified complexes) or γ TuSC differ from each of the homogenous versions? The results presented here are beginning to provide answers to some of these questions, but gaining a true understanding of how γ -tubulin complexes that differ only slightly in their composition are perhaps used to fine-tune microtubule organization will be the subject of future studies.

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