Xgrip109: A γ Tubulin–Associated Protein with an Essential Role in γ Tubulin Ring Complex (γTuRC) Assembly and Centrosome Function

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Abstract. Previous studies indicate that γ tubulin ring complex (γTuRC) can nucleate microtubule assembly and may be important in centrosome formation. γTuRC contains approximately eight subunits, which we refer to as *Xenopus* gamma ring proteins (Xgrips), in addition to γ tubulin. We found that one γTuRC subunit, Xgrip109, is a highly conserved protein, with homologues present in yeast, rice, flies, zebrafish, mice, and humans. The yeast Xgrip109 homologue, Spc98, is a spindle–pole body component that interacts with γ tubulin. In vertebrates, Xgrip109 identifies two families of related proteins. Xgrip109 and Spc98 have more homology to one family than the other. We show that Xgrip109 is a centrosomal protein that directly interacts with γ tubulin. We have developed a complementation assay for centrosome formation using demembranated *Xenopus* sperm and *Xenopus* egg extract. Using this assay, we show that Xgrip109 is necessary for the reassembly of salt-disrupted γTuRC and for the recruitment of γ tubulin to the centrosome. Xgrip109, therefore, is essential for the formation of a functional centrosome.

Properly organized microtubule arrays are critical for many cellular functions including mitosis, cytokinesis, and axonal and intracellular transport (for review see Kellogg et al., 1994). Microtubules are dynamic polymers that assemble from α-/β-tubulin heterodimers (Weisenberg, 1972; Mitchison and Kirschner, 1984). In vivo, microtubule dynamics are coordinately regulated by many cellular factors (for review see Desai and Mitchison, 1998). There are proteins that stabilize (for reviews see Vallee et al., 1984; Olmstead, 1986), destabilize (Endow et al., 1994; Belmont and Mitchison, 1996), or sever (McNally et al., 1996) microtubule polymers, as well as proteins that sort microtubules into different arrays. In addition, microtubule nucleation is temporally and spatially controlled within the cell, occurring primarily at structures called microtubule organizing centers (MTOCs)[1] (Kellogg et al., 1994). The major MTOC in animal cells is the centrosome that consists of a pair of centrioles surrounded by an electron-dense cloud of pericentriolar material (PCM). The PCM is responsible for microtubule nucleation (Kellogg et al., 1994).

The discovery of γ tubulin as a suppressor of a β-tubulin mutation in *Aspergillus nidulans* (Weil et al., 1986; Oakley and Oakley, 1989) was a major breakthrough in the study of microtubule nucleation at a molecular level. γ Tubulin is highly conserved and has been found in all eukaryotes examined (for review see Oakley, 1992). Most γ tubulins share over 60% amino acid identity, with the exception of *Saccharomyces cerevisiae* γ tubulin, which is only ~40% identical to the other γ tubulins (Sobel and Synder, 1995; Marschall et al., 1996; Spang et al., 1996). γ Tubulin is localized to all MTOCs such as the spindle–pole body (the major fungal MTOC) and the centrosome (Stearns et al., 1991; Zheng et al., 1991).

Genetic studies in *Aspergillus* (Oakley et al., 1990), *Saccharomyces pombe* (Horio et al., 1991), *Saccharomyces cerevisiae* (Sobel and Synder, 1995; Marschall et al., 1996; Spang et al., 1996), and *Drosophila melanogaster* (Sunkel et al., 1995; Tavosanis et al., 1997) have demonstrated that γ tubulin is an essential gene required for the assembly of a functional mitotic spindle. Antibody inhibition or depletion experiments performed in animal cells (Joshi et al., 1992) and in *Xenopus* egg extracts (Felix et al., 1994), respectively, further show the critical role of γ tubulin in microtubule nucleation at the centrosome.

Biochemical studies were also initiated to study how

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1. *Abbreviations used in this paper*: γTuRC, γ tubulin ring complex; Xgrip, *Xenopus* gamma ring protein; Asp, ammonium sulfate pellet; CSF, cytostatic factor; DAPI, 4′,6-diamidino-2-phenylindole; EST, expressed sequence tag; GST, glutathione S-transferase; MTOC, microtubule organizing center; ORF, open reading frame; PCM, pericentriolar material; PEG, polyethylene glycol.

γ tubulin is involved in microtubule nucleation at the MTOCs. Human γ tubulin translated in vitro is monomeric (Melki et al., 1993), and binds to microtubules in an end-specific manner (Li and Joshi, 1995). On the other hand, in animal cells, the γ tubulin that is not associated with the centrosome is found in large cytoplasmic complexes (Raff et al., 1993; Stearns and Kirschner, 1994). The purified *Xenopus* γ-tubulin–containing complex has an estimated molecular mass of over 2,000 kD. This complex, the γ tubulin ring complex (γTuRC), has an open ring structure and can nucleate microtubules in vitro. In addition to multiple γ-tubulin molecules, the γTuRC contains approximately eight additional polypeptides (Zheng et al., 1995). The *S. cerevisiae* γ tubulin also appears to form a complex with at least two other proteins: the spindle–pole body components Spc98 and Spc97 (Geissler et al., 1996; Knop et al., 1997). However, since this γ-tubulin complex has a much smaller S value (6 S) (Geissler et al., 1996; Knop et al., 1997) than that of the γTuRC (>25 S), it is not clear what the functional relationship is between these complexes or if the yeast γ-tubulin complex is able to nucleate microtubules in vitro. Based on the structure and function of the γTuRC, two models were proposed to explain how the γTuRC may nucleate microtubule assembly (Zheng et al., 1995; Erickson and Stoffler, 1996). One model suggests that the γTuRC acts as a seed, similar to the plus end of a microtubule, to nucleate microtubule assembly (Zheng et al., 1995). The other model proposes that the γTuRC unrolls into a filament, similar to a tubulin protofilament, to initiate microtubule polymerization (Erickson and Stoffler, 1996). Further biochemical and structural analyses are needed to study the mechanism of microtubule nucleation by the γTuRC.

In parallel structural studies, EM tomography has revealed hundreds of γTuRC-like rings embedded in the PCM of *Drosophila* and of the surf clam *Spisula* (Moritz et al., 1995*b*; Vogel et al., 1997); in *Drosophila* these rings are known to contain γ tubulin (Moritz et al., 1995*a*). The combined structural and biochemical studies of γ tubulin led to the hypothesis that the γTuRC is anchored within the PCM where it acts to nucleate microtubules (Zheng et al., 1995).

To better understand the relationship between the centrosomal γ-tubulin–containing rings and the cytosolic γTuRC, we have begun to characterize the γTuRC subunits. Here we report the identity and function of one of the subunits, Xgrip109. We show that Xgrip109 is a highly conserved centrosomal protein that is essential for γ-tubulin function.

Materials and Methods

Buffers

Buffers used were: Hepes 100 (50 mM Hepes, pH 8, 1 mM $MgCl_2$, 1 mM EGTA, 100 mM KCl); Hepes 300, Hepes 500, and Hepes 1 M are the same as Hepes 100 except that the concentration of KCl is 300 mM, 500 mM, and 1 M, respectively; cytostatic factor (CSF)-XB (10 mM potassium Hepes, pH 7.7, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, 5 mM EGTA, pH 7.7); BRB80 (80 mM potassium Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA); and OR2 (2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHPO_4$, 5 mM Hepes, final pH adjusted to 8.3).

Generating Mouse Polyclonal Ascites against the Xgrips

To obtain quantities of the Xgrips sufficient for mouse immunization, we performed a large-scale immunoprecipitation of the γTuRC from a 15–25% ammonium sulfate cut of a concentrated *Xenopus* CSF–arrested egg extract (Murray, 1991). Briefly, ammonium sulfate was added to 15% (from a 100% ammonium sulfate stock solution) to ~100 ml of concentrated extract that had been clarified by centrifugation at 35,000 rpm in a rotor (model SW55; Beckman Instruments, Inc., Palo Alto, CA) for 2 h. The extract was then centrifuged at 10,000 rpm in another rotor (model SS34; Sorvall Inc., Newtown, CT) for 15 min. The supernatant from this spin was adjusted to 25% ammonium sulfate and then the pellet was collected by centrifugation as before. The 25% ammonium sulfate pellet (Asp), which contained over 90% of γTuRC, was resuspended in 75 ml of Hepes 100 and then clarified by centrifugation at 30,000 rpm in a rotor (model SW55; Beckman Instruments, Inc.) for 1 h. The supernatant was collected and 2.6 ml of anti–γ-tubulin antibodies (XenC) (Zheng et al., 1995) at a concentration of 2 mg/ml were added. After a 1-h incubation at 4°C, 2.7 ml of settled protein A–agarose beads (Life Technologies, Inc., Gaithersburg, MD) was added and then the incubation was continued for another hour. The protein A–agarose beads were then washed batchwise three times with 10 vol each of Hepes 100, Hepes 300, and Hepes 100. The γTuRC was eluted batchwise using 1.4 M $MgCl_2$. The protein sample was precipitated by addition of trichloroacetic acid to 10%, neutralized, dissolved in SDS sample buffer, and then separated using preparative 10% SDS-PAGE. Gel slices containing either Xgrip195, 133, a mixture of 110 and 109, or a mixture of 75 kD (Zheng et al., 1995) proteins were homogenized and used to immunize mice according to Harlow and Lane (1988). We estimate that between 1 and 10 μg of the total protein was injected into each animal. Ascites fluid was induced after the fifth injection in mice that gave a positive response by Western analysis.

Internal Peptide Sequencing of the Xgrips

To obtain internal peptide sequences, the Xgrips were prepared as described above and separated on preparative 10% SDS-PAGE. After blotting to polyvinylidine difluoride membranes (Applied Biosystems, Inc., Foster City, CA), proteins were visualized by staining with Ponceau S. The membrane-immobilized proteins were reduced, S-carboxymethylated, and then digested in situ with *Achromobacter* protease I and endoproteinase Asp-N (Iwamatsu and Yoshida-Kubomura, 1996). Digested peptides were separated by reverse-phase HPLC using an Å column (model Wakosil-II AR C18 300, 2.0 × 150 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Amino acid sequencing was carried out with a gas-phase sequencer (model PPSQ-10; Shimadzu Corp., Tokyo, Japan).

DNA Cloning and Sequencing

The mouse polyclonal ascites that recognized Xgrip109 by Western blot analysis were used to screen a λZAP® cDNA library of *Xenopus* oocytes (Stratagene, La Jolla, CA) according to Sambrook et al. (1989), with modifications as described (Hirano and Mitchison, 1994). We screened 7×10^5 plaques and isolated two positive clones (clone 3-1 and clone 3-4) that had overlapping sequences. Because neither clone had a complete 5′ coding region, we rescreened the library using clone 3-4 as probe. 20 positives were obtained. One clone (p109–14) was longer than clone 3-4, but it still lacked a complete 5′ coding region.

To obtain the missing 5′ end, we carried out 5′ rapid amplification of cDNA ends (RACE) using a 5′/3′ RACE Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Three partially overlapping primers corresponding to the 5′ region of the known sequence of p109–14 were made: (*a*) gsp1 (5′-ACTAATCCCACTGCTGCCGAT-3′), (*b*) gsp2 (5′-CGGAATTCGATGCTTGCAGCATTCTGTGC-3′), and (*c*) gsp3 (5′-CGGAATTCTGCACACTGCACATTCCGATC-3′) for the initial reverse transcription of *Xenopus* mRNA (gsp1) and two rounds of PCR (gsp2 and gsp3). Pfu DNA polymerase (Stratagene) was used in both rounds of PCR reactions. A 650-bp–amplified DNA fragment was cloned, sequenced, and contained the complete 5′ coding region of Xgrip109 since the open reading frame (ORF) began with a methionine, which was preceded by an in-frame stop codon 48-bp upstream. The race product contained the 131 amino acids that were missing from the 5′ end of p109–14.

Expression of Recombinant Fusion Proteins, Production of Rabbit Antibodies, and Immunoblotting

We produced six fusion proteins between glutathione S-transferase (GST) and six different fragments of the Xgrip109. Only the fusion between GST and the 134–244-amino acid fragment of Xgrip109, p109-2, elicited rabbit

antibodies that worked for Western analysis, immunofluorescence, and immunodepletion. The primers 5′-CGGGATCCCACGAGGCCAGGCCACAGAGC-3′ and 5′-CGGAATTCTTCAACAGATCCACTTGAGTC-3′ were used to PCR-amplify this fragment of Xgrip109 using p10-4 as template. The resulting fragment was subcloned into the BamHI and EcoRI sites of pGEX-2TK (Pharmacia Biotech., Inc., Piscataway, NJ). To purify the p109-2 fusion protein, 1 liter of bacterial culture (BL21 lys.S) expressing p109-2 was pelleted and lysed in ~20 ml of Hepes 100 containing 0.1 mM of PMSF. After centrifugation, the clarified extract was incubated with ~5 ml of prewashed glutathione agarose (Sigma Chemical Co.) for 1 h at 4°C. The glutathione agarose was then packed into a PD-10 column (Pharmacia Biotech., Inc.), washed sequentially with 10 column volumes each of Hepes 100, Hepes 500, and Hepes 100, and then eluted with 10 mM of reduced glutathione (Sigma Chemical Co.) in Hepes 100. Each of these wash and elution buffers contained 0.1 mM PMSF.

The p109-2 fusion protein and a synthetic peptide (C)RLRVSMGTRGRRSFHV, corresponding to the COOH-terminal 16 amino acids of the Xgrip109 were used to immunize separate rabbits (Spring Valley Laboratories, Inc., Sykesville, MD). An NH_2-terminal cysteine was added to the peptide for sulfhydryl coupling. Peptide conjugation and antibody affinity purification were performed as described (Harlow and Lane, 1988). The antibodies against p109-2 fusion protein and the synthetic peptide are referred to as 109-2 and 109-c, respectively.

The XenC antibodies used in this paper were raised against a synthetic peptide corresponding to the last 15 amino acids of the *Xenopus* γ tubulin (Zheng et al., 1995). Immunoblotting was carried out using affinity-purified antibodies at a concentration of ~1 μg/ml using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Inc., Piscataway, NJ).

Preparation of Xenopus Egg Extracts and Demembranated Xenopus Sperm

Concentrated extracts made from CSF-arrested *Xenopus* eggs according to Murray (1991) were further clarified in a rotor (model TLS55; Beckman Instruments, Inc.) at 50,000 rpm, 4°C for 1 h. 20× energy mix (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, pH 7.7, 20 mM $MgCl_2$) was added to the supernatant to a final concentration of 1×. This extract was frozen in liquid N_2 and then stored at −80°C as 100-μl aliquots. All centrosome assembly assays were performed using the clarified and frozen extract. Demembranated *Xenopus* sperm was prepared as described (Sawin and Mitchison, 1991).

Centrosome Assembly Assay and Quantitation

1-μl of *Xenopus* sperm (~1.5×10^4 sperm/ml) that had been diluted 10-fold in CSF-XB was added to 10 μl of thawed and clarified *Xenopus* egg extract. After addition of 1 μl of 4 mg/ml rhodamine-tubulin (Hyman et al., 1991), the mixture was incubated at room temperature for 10 min. The reaction was stopped by diluting with 1 ml of BRB80 containing 30% glycerol (vol/vol) and then layered in a Corex (Corning GlassWorks, Corning, NY) tube onto 2 ml of BRB80 containing 30% glycerol, followed by spinning in a rotor (model HB-6; Sorvall Inc.) at 10,000 rpm for 20–30 min at 20°C onto a glass coverslip placed at the bottom of the tube (Evans et al., 1985). The sperm nuclei and microtubule asters were fixed in −20°C methanol for 5 min followed by hydration in BRB80 containing 0.1% Triton X-100 for 5 min. The samples were then stained with 0.2 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) dissolved in BRB80 containing 0.1% Triton X-100 for 2 min followed by mounting in antifade (1 mg/ml *p*-phenylenediamine, 0.5× PBS, pH 9, 50% glycerol, 0.02% NaN_3).

A total of 100 sperm nuclei were scored in random fields. These 100 sperm nuclei were divided into three groups: (*a*) sperm nuclei with a microtubule aster attached at the tip, "centrosome"; *(b)* sperm nuclei with a few disorganized microtubules attached at the tip, "disorganized centrosome"; or *(c)* sperm nuclei alone. Because the microtubule asters break away from the tips of the sperm nuclei at a low frequency, we also counted the free microtubule asters while scoring the sperm nuclei. The number of the free microtubule asters was subtracted from the sperm nuclei alone group and added to the sperm nuclei with a microtubule aster centrosome group.

30% Ammonium Sulfate Fractionation, Immunodepletion, and Complementation Assays

To fractionate the clarified egg extract with 30% ammonium sulfate, 43 μl of 100% ammonium sulfate was added to 100 μl of extract and then incubated on ice for 15 min, followed by centrifugation in a rotor (model SS34; Sorvall Inc.) at 10,000 rpm for 10 min at 4°C. The pellet, which contained over 90% of the total γTuRC and only ~20% of the total extract protein, was resuspended in 100 μl of either Hepes 100 or Hepes 1 M containing 1 mM GTP and then incubated on ice for 80 min. This mixture was either analyzed by sucrose gradient sedimentation (see below) directly or after desalting using a 1-ml P6 Bio-Spin column (Bio-Rad Laboratories, Hercules, CA), equilibrated in Hepes 100 containing 1 mM of GTP. The eluate was either analyzed by sucrose gradient sedimentation or concentrated ~20-fold by placing in a collodion bag (Sartorius, Göttingen, Germany) that was kept on a bed of Sephadex G-50 resin (Pharmacia Biotech., Inc.) at 4°C. The concentrated eluate was then used in complementation assays (see below).

To immunodeplete Xgrip109 and γ tubulin from the resuspended 30% Asp in either Hepes 100 or Hepes 1 M, 10 μl of 109-2 IgG (~0.25 mg/ml), 4.5 μl of XenC IgG (~1.6 mg/ml), or 2.5 μl of random IgG (~3 mg/ml) were bound to 10–20 μl of settled protein A beads. The beads were washed with either Hepes 1 M or Hepes 100 and then added to the appropriate resuspended pellets that were incubated on ice for 20 min. After rotating for 1 h at 4°C, the protein A beads were collected by pelleting and then washed. The immunoprecipitated proteins were analyzed by SDS-PAGE followed by Coomassie blue staining or Western analysis, probing with XenC and 109-2. To estimate the percentage of total γ tubulin that remained bound to Xgrip109 in high salt, the amount of γ tubulin immunoprecipitated using XenC in low-salt buffer (this represents the total γ tubulin) was compared with the amount of γ tubulin immunoprecipitated using 109-2 in high salt buffer by densitometry scanning. The supernatants from the 109-2 and random IgG immunodepletion reactions were desalted and concentrated ~20-fold as described above and then used for complementation assays (see below). To assess whether γ tubulin and Xgrip109 interact with each other, the immunoprecipitation was carried out with an excess of extract. Under this condition, the amount of γ tubulin present in the extract is in excess of the added γ-tubulin antibodies and results in cleaner immunoprecipitation.

To prepare γTuRC-depleted egg extract, either 4.5 μl of XenC antibodies (~1.6 mg/ml), or 2.5 μl of control random IgG (~3 mg/ml) were bound to 10–20 μl of settled Affi-Prep Protein A Support (Bio-Rad Laboratories). 100 μl of the thawed *Xenopus* egg extract was added to the CSF-XB–washed protein A beads and then incubated with rotation at 4°C for 1 h. The protein A beads were then pelleted and the supernatant was used for centrosome assembly assays.

To determine whether the random IgG- or XenC IgG–depleted extracts were functional in centrosome assembly, 7.5 μl of each extract was combined with 2.5 μl of Hepes 100 containing 1 mM of GTP, 1 μl of rhodamine-tubulin, and 1 μl of *Xenopus* sperm (see above). The centrosome formation assay was carried out as described above. To complement the XenC-depleted extract, 2.5 μl of the concentrated Asp that was resuspended in Hepes 100, Hepes 1 M, Hepes 1 M depleted with random IgG, or Hepes 1 M depleted with 109-2 IgG was added to 7.5 μl of XenC-depleted extract instead of buffer. Centrosome formation was quantitated as described above.

Cell Culture and Cell Extract

Cell line XLK-WG, derived from a primary culture of *Xenopus* kidney cells, was provided by Z. Wu and J.G. Gall (both from Carnegie Institution of Washington, Baltimore, MD). The cells were grown at 32°C in a water-saturated atmosphere of 5% CO_2 in air in medium containing 60% RPMI medium 1640 (Life Technologies, Inc.), 20% heat-inactivated FBS (Life Technologies, Inc.), 100 U/ml each of penicillin and streptomycin, and 2 g/liter of $NaHCO_3$.

To make XLK-WG cell extract, 5 plates (100-mm-diam) of 80% confluent XLK-WG cells were first rinsed with Hepes 100 and then harvested by scraping. The cell pellet was resuspended in 1 ml Hepes 100 containing 0.1% Triton X-100, 1 mM 2-mercaptoethanol, 1 mM PMSF, 0.01 mM benzamidine HCl, 0.001 mg/ml phenanthroline, and 0.01 mg/ml each of aprotinin, leupeptin, and pepstatin A. The cells were homogenized in a Dounce homogenizer, frozen in liquid N_2, and then stored at −80°C.

Immunofluorescence Staining

For indirect immunofluorescence staining, XLK-WG cells were rinsed with OR2, fixed in −20°C methanol for 5 min, followed by hydrating in OR2 for 5 min. After permeabilizing with OR2 containing 0.1% Triton

X-100 for 1 min, the cells were rinsed with OR2, blocked in OR2 containing 2% BSA for 30 min, and then incubated for 1 h with monoclonal anti–γ-tubulin antibody diluted 200-fold (clone number GTU-88; Sigma Chemical Co.) and 109-2 or 109-c diluted 400-fold. After three 5-min washes, the cells were incubated for 1 h with FITC-conjugated goat anti–mouse and Cy3-conjugated goat anti–rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 400-fold. After three 5-min washes, the cells were stained with DAPI and then mounted in antifade.

To localize γ tubulin and Xgrip109 in the sperm nuclei and microtubule asters, we carried out the centrosome assembly assay as described above but in the absence of rhodamine tubulin. The sperm nuclei and microtubule asters were centrifuged onto round glass coverslips (refer to above) and the same immunofluorescence staining steps as for the XLK-WG cells were performed. Photomicrographs were obtained using a cooled charge-coupled device camera (Princeton Scientific Instruments, Inc., Princeton, NJ) on a microscope (model E800; Nikon Corp., Tokyo, Japan). Images were handled digitally using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Sucrose Gradient Sedimentation

To analyze the sedimentation behavior of γ tubulin and Xgrip109 in XLK-WG cell extracts and *Xenopus* egg extracts, 5 ml of 5–40% sucrose step gradients were poured and allowed to diffuse overnight. Each step (950 μl) contained 5, 10, 20, 30, or 40% sucrose in Hepes 100. The thawed XLK-WG cell extract was clarified at top speed in a microfuge (Eppendorf 5417C; Hamburg, Germany) for 10 min, and 300 μl of this supernatant or 300 μl of clarified egg extract was directly loaded onto the sucrose gradients. Another 300 μl of the supernatant or clarified egg extract was precipitated with 2.5% polyethylene glycol (PEG), mol wt of 8,000 (Sigma Chemical Co.). The pellet, which contained over 90% of the total cellular γTuRC, was resuspended in 300 μl of Hepes 100 and then loaded onto a separate gradient. Centrifugation was performed in an SW55 rotor (model SW55; Beckman Instruments, Inc.) at 50,000 rpm for 4.5 h at 4°C. 300-μl fractions were collected from the top of each gradient. The fractions were separated by 10% SDS-PAGE and then analyzed by Western analysis, probing with anti–γ-tubulin and anti-Xgrip109 antibodies.

We used 2 ml of 5-40% sucrose gradients to analyze 100 μl of the Asp that was resuspended in either Hepes 100 or Hepes 1 M (see above). The gradients were poured as described above except that each of the five steps was 400 μl. The standards, BSA (4.4 S), bovine liver catalase (11.3 S), and porcine thyroglobulin (19.4 S), were dissolved in either Hepes 100 or Hepes 1 M and run on identical sucrose gradients as the samples. The gradients were centrifuged in a rotor (model TLS55; Beckman Instruments, Inc.) at 55,000 rpm for 2 h at 4°C. 130-μl fractions were collected from the top of each gradient and analyzed by SDS-PAGE followed by Western analysis, probing with anti–γ-tubulin and anti-Xgrip109 antibodies. Because the Hepes 1 M resuspended samples were heavier than the 5% sucrose at the top of the gradient, standards dissolved in Hepes 1 M appeared to sediment faster than the same standards dissolved in Hepes 100.

Results

Xgrip109 Is a Conserved Protein

We previously reported the purification and biochemical characterization of γTuRC (Zheng et al., 1995). γTuRC consists of approximately eight uncharacterized polypeptides in addition to γ tubulin. We refer to each polypeptide as Xgrip followed by the respective apparent molecular mass of that polypeptide (see Fig. 3 *B*). We took two approaches to identify the Xgrips. First, we raised mouse polyclonal antibodies against gel-purified Xgrips. Xgrip109 and Xgrip195 both elicited antibody responses in mice. Second, we sequenced the purified Xgrips that were immobilized on polyvinylidine difluoride membrane. We obtained internal peptide sequences for Xgrip109, Xgrip110, and the Xgrip75s. Since we could not resolve Xgrip109 and Xgrip110 well enough on the gel to sequence them separately, the peptide sequences obtained are derived from both proteins, and the same is true for the Xgrip75 group of proteins.

We screened a λZAP *Xenopus* oocyte cDNA library using the mouse polyclonal antibodies against Xgrip109 and cloned overlapping cDNAs that contained a single ORF (refer to Materials and Methods). We found that half of the peptide sequences we obtained had perfect matches in the ORF, confirming that we cloned the correct Xgrip109 cDNA. We believed that the peptide sequences that do not match Xgrip109 are derived from Xgrip110. The longest cDNA clone has a stop codon followed by a poly A tail at its 3′ end, but it lacks a complete 5′ coding sequence. Using 5′ RACE, we cloned the missing 5′ end.

The complete Xgrip109 cDNA (GenBank/EMBL/DDBJ accession number AF052663) encodes a protein of 906 amino acids with a predicted molecular mass of 103.6 kD. Searches of the protein databases reveal that Xgrip109 is most closely related to Spc98, a *S. cerevisiae* spindle–pole body component (Geissler et al., 1996). Although overall Xgrip109 is only ~21% identical and 46% similar to Spc98, the stretch of amino acids (~180) in the middle of both proteins share 28% amino acid identity (Fig. 1). When we searched the expressed sequence tag (EST) databases using the Xgrip109 sequence, we found two groups of conserved human, mouse, and zebrafish ESTs that both share homology with a region of Xgrip109 between amino acids 512–684. The first group of ESTs (Fig. 2 *A*) are over 85% identical to Xgrip109 over the entire region between amino acids 512–684. The second group of ESTs, consisting of one human EST and one mouse EST that are over 85% identical to each other, shares more limited homology (~30% identity) with Xgrip109 over a smaller region between amino acids 534–612. Furthermore, database searches with the entire Xgrip109 sequence also identified homologous ESTs in rice (~52% amino acid identity, accession number C26482) and *Drosophila* (36% amino acid identity, accession number AA246343). These sequence analyses suggest that Xgrip109 is a conserved protein that, at least in vertebrates, may share homology with two families of related proteins.

Xgrip109 Is a Component of the γTuRC

To study the function of Xgrip109, we raised antibodies against a GST fusion protein with amino acids 134–244 of Xgrip109, as well as a synthetic peptide corresponding to the COOH terminus of Xgrip109 (refer to Materials and Methods). The two affinity-purified antibodies, which we refer to as 109-2 and 109-c, respectively, specifically recognize a protein of 109 kD when used to probe Western blots of *Xenopus* egg extracts (Fig. 3 *A*).

Two approaches were used to confirm that Xgrip109 is indeed a component of the γTuRC. First, we compared the patterns of proteins coimmunoprecipitated by antibodies against Xgrip109 (109-2) and γ tubulin (XenC, Fig. 3 *B*). Both XenC (an antibody raised against the COOH terminus of γ tubulin (Zheng et al., 1995) and 109-2 antibodies immunoprecipitated the same set of proteins. Moreover, this protein profile is identical to that of the γTuRC that we identified previously (Zheng et al., 1995). Although immunoprecipitations from clarified egg extracts gave a much higher background than immunopre-

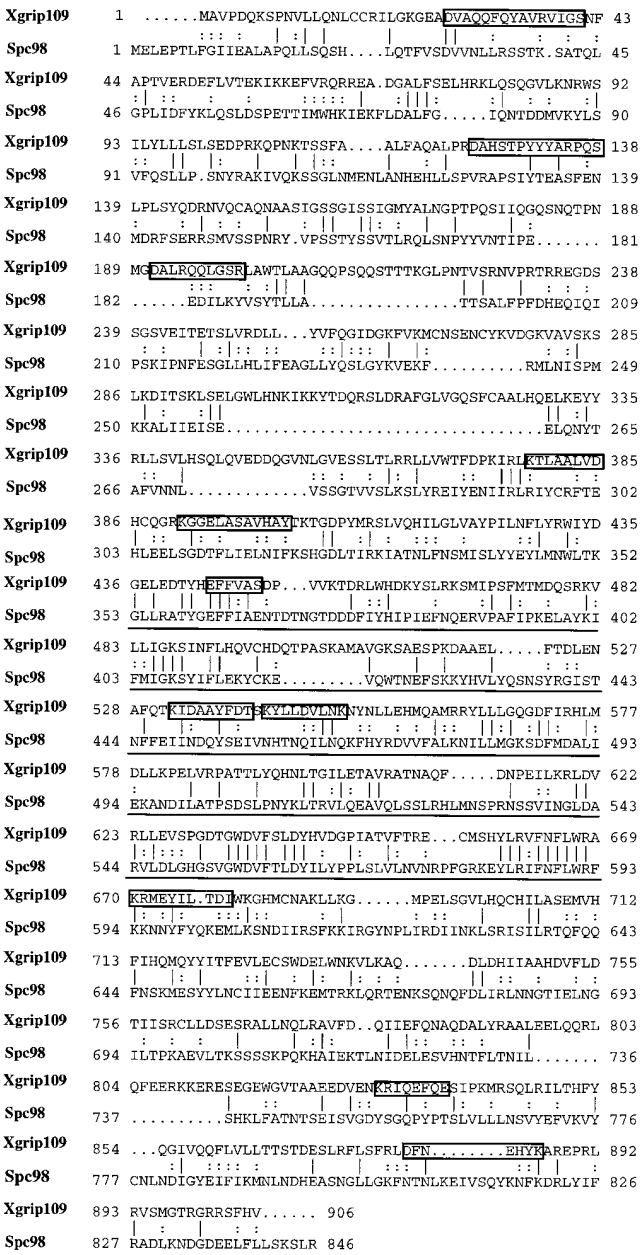

Figure 1. Xgrip109 is homologous to the yeast γ-tubulin–interacting protein Spc98. Sequence comparison of Xgrip109 and Spc98. *Vertical lines*, identical amino acids; *two dots*, conserved amino acid changes. The overall amino acid identity shared between the two sequences is ~21%. The underlined region shares ~28% amino acid identity. The sequences in Xgrip109 that match the internal peptide are boxed.

cipitations from resuspended, clarified 30% Asps, the γTuRC components are still readily identifiable (Fig. 3 *B*). The presence of γ tubulin and Xgrip109 in the immunoprecipitates was confirmed by Western analysis using XenC and 109-2, respectively (data not shown).

Second, we compared the sedimentation behavior of Xgrip109 and γ tubulin on sucrose gradients. *Xenopus* egg extracts or cell extracts made from a *Xenopus* kidney cell line were either directly sedimented (data not shown) on 5–40% sucrose gradients, or first precipitated with 2.5%

Figure 2. There may be two protein families in vertebrates that share homology with Xgrip109. (*A*) Sequence comparisons among one group of human (GenBank/EMBL/DDBJ accession number T55505), mouse (accession number AA152700), zebrafish (accession number AA495279) ESTs, and Xgrip109 (from amino acid 512 to 684). These sequences share over 85% amino acid identity. (*B*) Sequence comparisons among another group of human (accession number R13714) and mouse (accession number AA543491) ESTs, and Xgrip109 (from amino acid 534 to 612). Although the two EST sequences share over 85% amino acid identity with each other, Xgrip109 is only ~38% identical to the two EST sequences.

PEG and then sedimented (Fig. 3 *C*). The fractions from these gradients were analyzed by Western blotting, probing with antibodies against Xgrip109 and γ tubulin. We found that Xgrip109 and γ tubulin cosediment (Fig. 3 *C*). These results show that Xgrip109 is a component of the γTuRC.

Xgrip109 Is Localized to the Centrosomes

Since Xgrip109 is a component of the γTuRC, we expected Xgrip109, like γ tubulin, to localize to centrosomes. Double label–immunofluorescence staining for γ tubulin and Xgrip109 in *Xenopus* kidney tissue culture cells (XLK-WG) revealed that Xgrip109, like γ tubulin, does indeed localize to centrosomes (Fig. 4 *A*).

We next assembled centrosomes in vitro by incubating demembranated *Xenopus* sperm with *Xenopus* egg extracts. In the presence of an ATP-containing energy regenerating system, the pair of centrioles located at the tips of the sperm nuclei can recruit proteins to form a functional centrosome that nucleates a microtubule aster (Felix et al., 1994; Stearns and Kirschner, 1994). Using immunofluorescence staining, we found that, like γ tubulin, Xgrip109 also localized to these in vitro assembled centrosomes (Fig. 4 *B*). To make sure that the γ tubulin and Xgrip109 staining would not bleed through due to microtubule staining, we omitted the microtubule label and visualized aster formation by phase-contrast microscopy. The extensive microtubule arrays appear clearly in the phase image (Fig. 4 *B, arrows*).

Xgrip109 Directly Interacts with γ Tubulin in the γTuRC

To determine if the interaction between Xgrip109 and γ

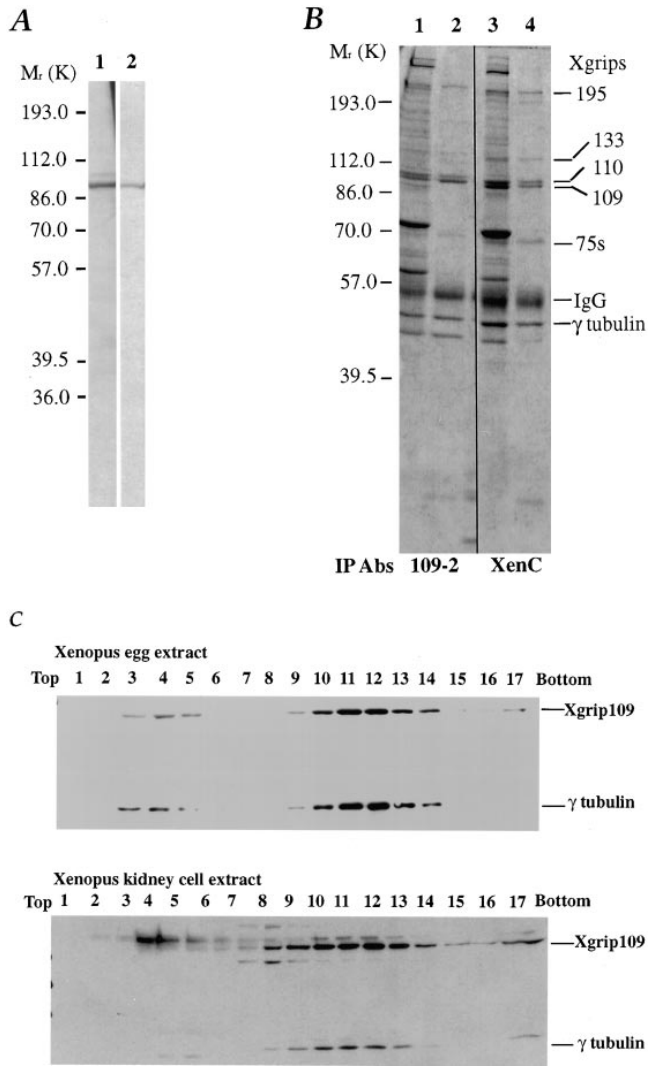

Figure 3. Xgrip109 is a component of γTuRC. (*A*) Xgrip109 antibodies. Lanes *1* and *2* show Western analysis of *Xenopus* egg extracts separated by 10% SDS-PAGE followed by immunoblotting with affinity-purified antibodies 109-2 and 109-c, respectively. Both antibodies specifically recognize a protein of 109 kD in the *Xenopus* egg extract. (*B*) Antibodies against Xgrip109 (*109-2*) and γ tubulin (*XenC*) both immunoprecipitated γTuRC components. Lanes *1* and *3* are immunoprecipitations carried out from the clarified egg extracts. Lanes *2* and *4* are immunoprecipitations carried out from 30% ammonium sulfate precipitates that were resuspended in low-salt buffer (refer to Materials and Methods). The immunoprecipitated proteins were separated by 10% SDS-PAGE and stained with Coomassie blue. Each of the γTuRC components (Xgrips) is indicated by its respective molecular mass. Immunoprecipitating antibodies are indicated at the bottom of the lanes. (*C*) Xgrip109 cosediments with γ tubulin. *Xenopus* egg extracts or extracts made from a *Xenopus* kidney cell line (XKL-WG cell line) were precipitated with 2.5% PEG, a treatment that precipitates more than 90% of the γ tubulin (data not shown). The pellet was resuspended in Hepes 100 and then sedimented on a 5–40% sucrose density gradient. Fractions were collected from the top of the gradients and then analyzed by Western blotting, probing with XenC and 109-2. The last fraction (fraction *17*) in each sedimentation experiment included the pellet.

tubulin is direct, we disrupted the γTuRC with high salt and examined the sedimentation behavior of Xgrip109 and γ tubulin by sucrose gradient sedimentation. The γTuRC was precipitated from the *Xenopus* egg extract with 30% ammonium sulfate and resuspended in either low-salt or high-salt buffers and then incubated on ice for 60–80 min to allow complete dissociation of γTuRC in the presence of high salt. Both samples were then separated on 5–40% sucrose gradients followed by Western analysis, probing with antibodies against Xgrip109 and γ tubulin. Protein standards BSA, catalase, and thyroglobulin were dissolved in either low-salt or high-salt buffers and then run on identical gradients. Fig. 5 *A* shows that high salt caused both γ tubulin and Xgrip109 to migrate as a smaller complex (~11 S). On the other hand, the γTuRC remained intact (>19.4 S) when the 30% Asp was resuspended in the low-salt buffer (Fig. 5 *A*).

To determine whether the salt-dissociated Xgrip109 remained associated with any other γTuRC components, we immunoprecipitated Xgrip109 from the 30% Asp resuspended in either low-salt or high-salt buffers. We found that γ tubulin was the only γTuRC subunit that coimmunoprecipitated with Xgrip109 in high salt (Fig. 5 *B,* lane *2*). The presence of Xgrip109 and γ tubulin in the immunoprecipitates was confirmed by Western analysis (data not shown). These results suggest that Xgrip109 directly binds to γ tubulin in γTuRC. We estimated, based on densitometry scanning, that less than half of the total γ tubulin in the γTuRC remained associated with Xgrip109 in the high-salt buffer (refer to Materials and Methods). As a control, γ tubulin was also immunoprecipitated in parallel using XenC. Since the XenC antibody did not bind to γ tubulin well in high-salt buffers (Fig. 5 *B,* lane *1*), we could not determine whether the remaining γ tubulin binds to any other Xgrips under high salt conditions.

Immunodepletion of Xgrip109 Blocks γTuRC Reassembly

Since we were able to disrupt the γTuRC with high salt, we wanted to test whether removing the salt would allow the γTuRC to reform. Furthermore, if the γTuRC could reassemble, we wanted to examine whether Xgrip109 is needed for γTuRC reformation. We used sucrose gradient sedimentation to analyze what happened to the γTuRC after salt treatment and immunodepletion with either random IgG or Xgrip109 IgG. A 30% Asp was resuspended in high-salt buffer and then immunodepleted with either random IgG or Xgrip109 antibodies (109-2). The immunodepletion supernatants were either directly loaded on 5–40% sucrose gradients, or desalted, and then loaded on sucrose gradients. We analyzed the sucrose gradient fractions by Western, probing with anti–γ-tubulin and anti-Xgrip109 antibodies. The control, Fig. 6 *E*, shows that under low-salt conditions, the intact γTuRC sedimented as a large particle (>19.4 S). On the other hand, the γTuRC was completely dissociated when the 30% Asp was resuspended in high-salt buffer and then immunodepleted with either random IgG (Fig. 6 *A*) or Xgrip109 IgG (Fig. 6 *B*). When the salt was removed from the random IgG-depleted sample, at least 50% of γ tubulin and Xgrip109 were assembled into a complex that had the same S value as that

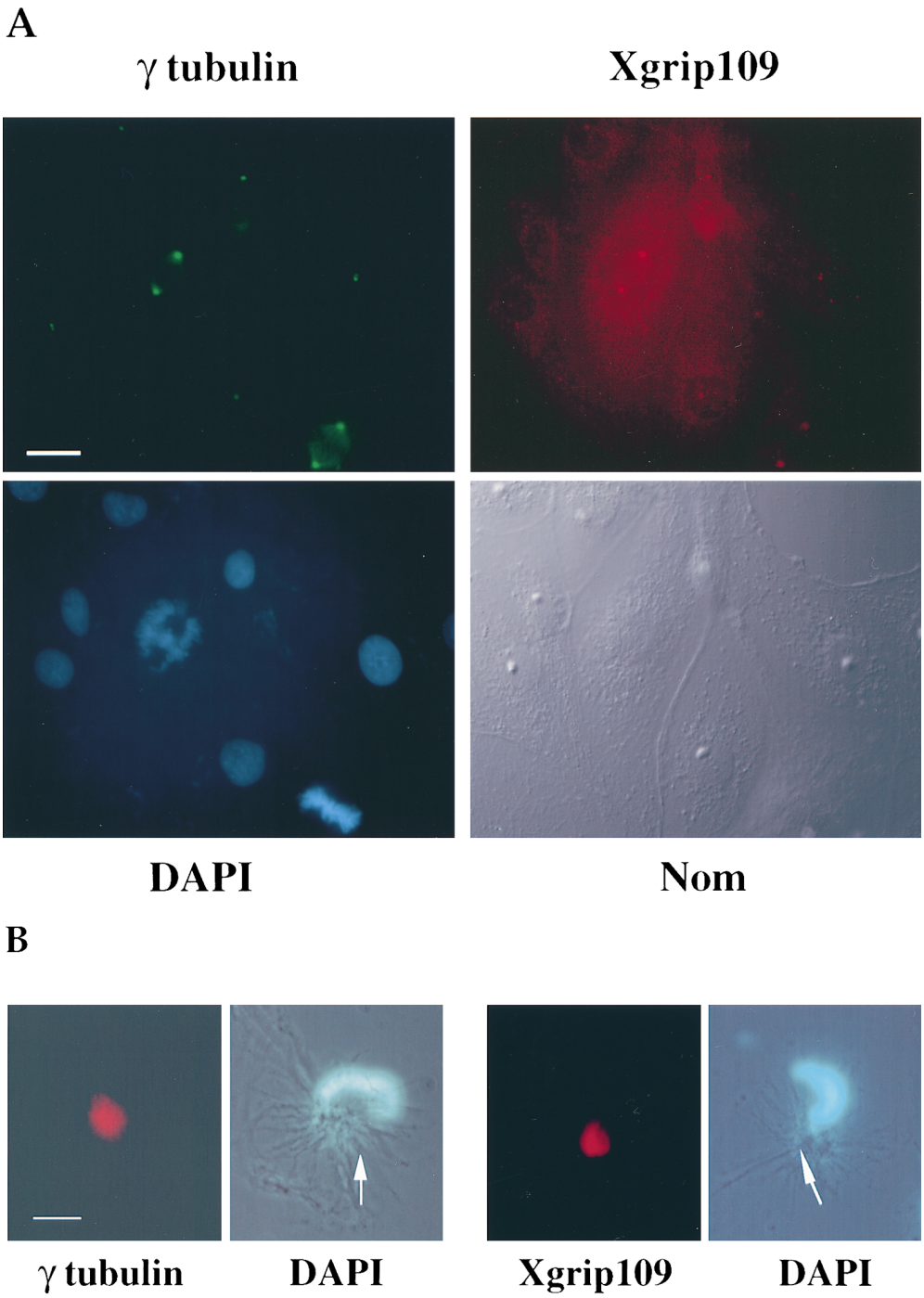

Figure 4. Xgrip109 is localized to centrosomes. (*A*) Xgrip109 and γ tubulin colocalized to the centrosomes in XLK-WG cells. *γ tubulin*, γ-tubulin localization revealed by fluorescein secondary antibody; *Xgrip109*, Xgrip109 localization revealed by Cy3 secondary antibody; *DAPI*, DNA staining with DAPI; *Nom,* Nomarski images of the cells. (*B*) Xgrip109 is localized to the in vitro–assembled centrosomes. The in vitro–assembled centrosomes were spun onto glass coverslips and indirect immunofluorescence staining was carried out using anti–γ-tubulin and anti-Xgrip109 antibodies (refer to Materials and Methods). *γ tubulin*, γ tubulin was localized to the tip of the sperm nucleus; *Xgrip109*, Xgrip109 was also localized to the tip of the sperm nucleus; *DAPI*, the two-sperm nuclei that were stained with either anti–γ-tubulin antibody (GTU-88) or anti-Xgrip109 antibodies (109-2) were stained with DAPI. The images are a combination of fluorescence and phase images. *Arrows*, microtubule asters at the tips of the two-sperm nuclei. Bars: (A) 20 μm; (B) 10 μm.

of the endogenous γTuRC (Fig. 6 *C*). When the salt was removed from the Xgrip109–depleted sample, the remaining γ tubulin had a similar S value to that in high-salt (Fig. 6 *D*). We analyzed the proteins that were removed by 109-2 IgG and random IgG by both Coomassie blue staining and Western, probing with 109-2 and XenC (Fig. 6, *F* and *G*). It is clear that, compared with random IgG, 109-2 IgG specifically removed Xgrip109 and γ tubulin. These results suggest that 50% of γTuRC reassembled after desalting. Furthermore, removing Xgrip109 and a small amount of γ tubulin from the resuspended 30% Asp before lowering the salt concentration blocked the reassembly.

A Complementation Assay for Centrosome Assembly

To study the role of Xgrip109 (or any other Xgrips) in the recruitment of γ tubulin to the centrosome and/or the formation of a functional centrosome, we needed to develop a centrosome formation assay where the activity is dependent on a source of γTuRC that we can biochemically manipulate to selectively remove specific Xgrips. Since we found that the γTuRC present in a 30% Asp can be dissociated with salt and reformed by desalting, we hope to use this as a source of γTuRC.

We took advantage of the existing centrosome formation assay using *Xenopus* egg extracts and sperm (Felix et al.,

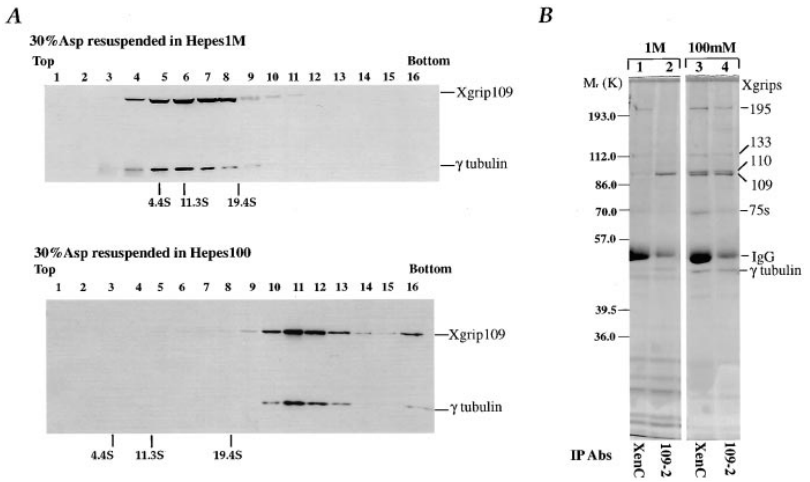

Figure 5. Xgrip109 interacts directly with γ tubulin. (*A*) γTuRC is dissociated in high salt. Clarified *Xenopus* egg extracts were first precipitated with 30% ammonium sulfate. The pellet (*30% Asp*) was resuspended in either Hepes 1 M (refer to Materials and Methods) or Hepes 100. The resuspended proteins were fractionated on 5–40% sucrose gradients. The sucrose gradient standards used were bovine serum albumin (*4.4S*), bovine liver catalase (*11.3S*), and bovine thyroglobulin (*19.4S*). The protein standards used (indicated at the bottom of each panel in *A*) were dissolved in either Hepes 1 M or Hepes 100 and then fractionated under identical conditions. Gradient fractions were collected from the top and each fraction was analyzed by SDS-PAGE followed by Western blotting with XenC and 109-2 antibodies. (*B*) A fraction of the γ tubulin remains associated with Xgrip109 in high salt. Immunoprecipitations with XenC (lanes *1* and *3*) or 109-2 antibodies (lanes *2* and *4*) were carried out using 30% Asp resuspended in either Hepes 1 M (lanes *1* and *2*) or Hepes 100 (lanes *3* and *4*). The precipitated proteins were separated by SDS-PAGE and then stained by Coomassie blue. The γTuRC components (*Xgrips*) are indicated by their respective molecular masses.

1994; Stearns and Kirschner, 1994). This centrosome formation assay was based on the original observation of Lohka and Masui (1983) who showed that the demembranated sperm and *Xenopus* egg extract can be used to study various cellular processes such as DNA replication, chromosome condensation, and centrosome and spindle formation in vitro. The centrosome formation assay is essentially an assay for PCM assembly around the sperm centrioles located at the tip of the sperm nucleus. When the sperm is incubated with the egg extract, PCM assembles around the centriole pair, resulting in the formation of a functional centrosome that can nucleate microtubule asters. Using this assay, Felix et al. (1994) showed that immunodepleting γTuRC from the egg extract blocked the

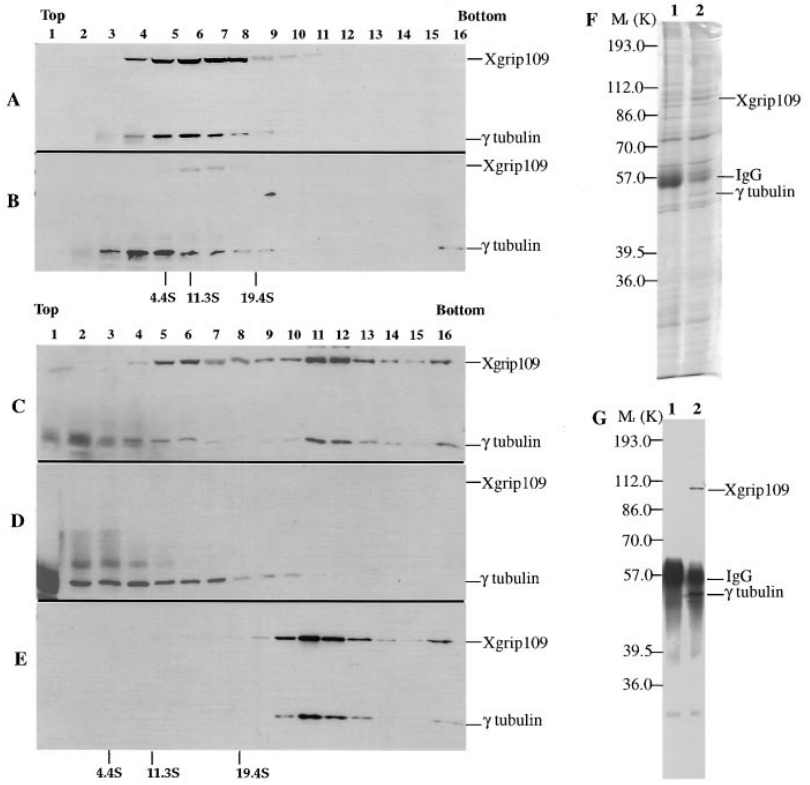

Figure 6. Xgrip109 is required for the reformation of salt-disrupted γTuRC. Clarified *Xenopus* egg extracts were precipitated with 30% ammonium sulfate. The pellet fraction (30% Asp) was resuspended in either Hepes 100 as control or in Hepes 1 M. Sucrose gradients in *A–E* are all 5–40%. (*A*) 30% Asp resuspended in Hepes 1M was immunoprecipitated with random IgG and then analyzed on a sucrose gradient. (*B*) The same as in *A*, except that the anti-Xgrip109 antibody, 109-2, was used in the immunoprecipitation. (*C*) The same as in *A*, except that after immunoprecipitation, a desalting step was included before the sucrose gradient sedimentation. (*D*) The same as in *B*, except that after immunoprecipitation, a desalting step was included before the sucrose gradient sedimentation. (*E*) Control, 30% Asp resuspended in Hepes 100, desalted, and fractionated on a sucrose gradient. (*F*) SDS-PAGE separation followed by Coomassie blue staining of proteins that were immunoprecipitated with random IgG (lane *1*) and 109-2 IgG (lane *2*). (*G*) The same protein samples in *F* were analyzed by Western probing with XenC and 109-2. Because samples in *A* and *B* contained higher amounts of salt than that of the samples in *C*, *D*, and *E* (refer to Materials and Methods), the sucrose gradient fractions of *A* and *B* cannot be compared directly to that of *C*, *D,* and *E*. The molecular weight standards for *A* and *B* are indicated at the bottom of *A*; *C*, *D*, and *E* are indicated at the bottom of *E*. Standards used are BSA (*4.4S*), bovine liver catalase (*11.3S*), and bovine thyroglobulin (*19.4S*).

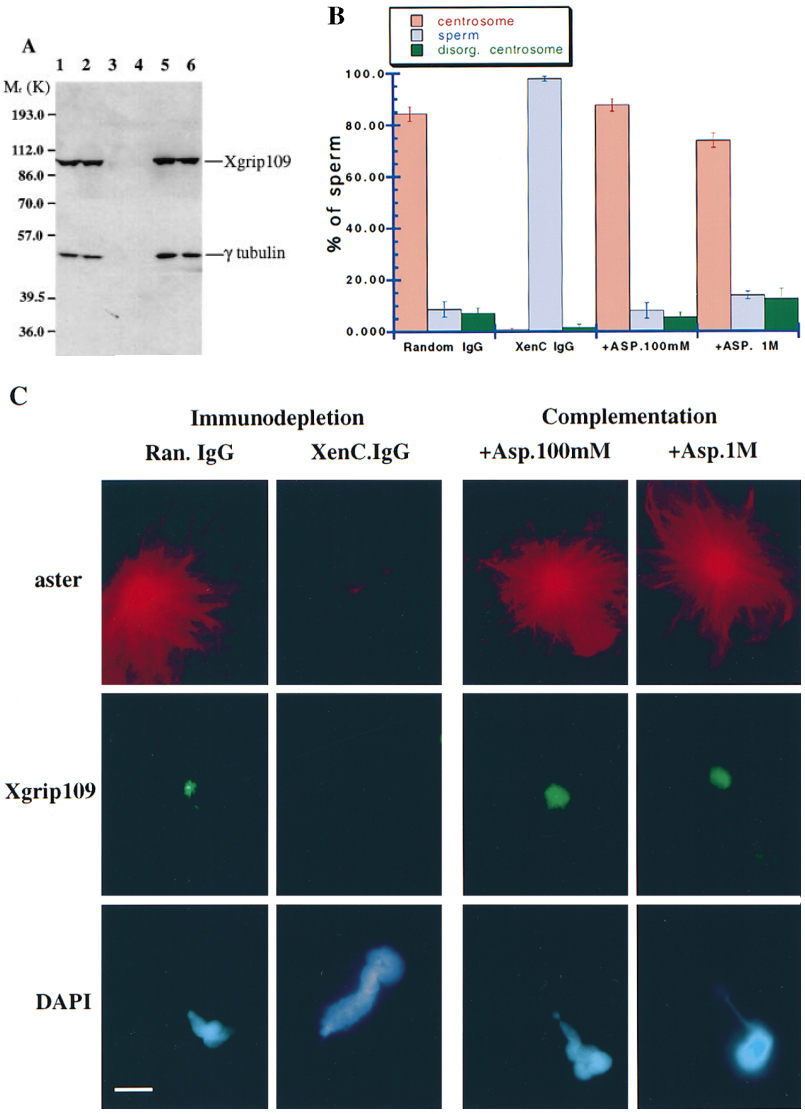

Figure 7. The complementation assay for centrosome formation. (*A*) Western analysis of the immunodepleted extracts, 30% ammonium supernatant, the pellet was probed with 109-2 and XenC. Lanes *1–4*: clarified extract; clarified extract immunodepleted using random IgG; clarified extract immunodepleted using XenC; 30% ammonium sulfate supernatant (there is no detectable γ tubulin or Xgrip109), respectively. Lanes *5* and *6*: 30% Asp resuspended in Hepes 100 (lane *5*) or Hepes 1 M (lane *6*), desalted, and then concentrated ~20-fold. (*B*) Quantitation of the complementation assays. *Red columns*, percentage of sperm with centrosomes that nucleated astral microtubules; *blue columns,* percentage of sperm without any microtubule nucleation from the tip of the sperm; *green columns*, percentage of sperm with centrosomes that nucleated a few disorganized microtubules; *Ran. IgG,* depletion with random IgG allowed over 80% of sperm centrioles to assemble into centrosomes; *XenC IgG*, depletion with XenC IgG completely abolished the centrosome assembly activity in the extract; *+ASP.100mM*, addition of 30% Asp resuspended in Hepes 100 to the γTuRC-depleted extract resulted in over 80% of sperm centrioles to assemble into centrosomes; *+ASP.1M*, addition of 1 M salt-treated and desalted 30% Asp to the γTuRC-depleted extract resulted in over 70% of sperm centrioles to assemble into centrosomes. The error bars were determined from four independent assays in each case. (*C*) Representative sperm nuclei with or without a microtubule aster from the assays in *B* are shown. The microtubules were labeled by the addition of a small amount of rhodamine-tubulin in the assays. Xgrip109 was detected using 109-c antibodies and a fluorescein conjugated goat anti–rabbit secondary antibody. The sperm DNA was stained by DAPI. Bar, 10 μm.

formation of a functional centrosome. We wanted to test whether the γTuRC present in the 30% Asp can complement the γTuRC-depleted extract to form a functional centrosome. We have previously found that the pellet alone did not support centrosome formation (data not shown).

Felix et al. (1994) used crude *Xenopus* egg extract for their centrosome formation assays. We found that clarifying the crude egg extract by centrifugation did not affect centrosome formation activity. Using XenC, we immunodepleted the γTuRC from the clarified extract (Fig. 7 *A*, lane *3*). Consistent with previous results (Felix et al., 1994), this γTuRC-depleted extract was not able to assemble a functional centrosome judging by the absence of a microtubule aster (Fig. 7 *B*). As expected, Xgrip109 was also absent from the centrosome (Fig. 7 *C*). The control extract that was depleted with random IgG, on the other hand, assembled a functional centrosome around the sperm centrioles, and Xgrip109 was present at the centrosome (Fig. 7, *B* and *C*).

To test whether the γTuRC present in the 30% Asp can complement the γTuRC-depleted extract to form a centrosome, we resuspended this pellet in low-salt buffer, desalted and concentrated it ~20-fold. We found that this γTuRC-containing fraction restored the centrosome assembly activity of the γTuRC-depleted extract (Fig. 7). We next tested whether an Asp that was resuspended in high salt still retains the complementing activity after salt removal. The pellet was resuspended in high-salt buffer and incubated on ice to allow complete dissociation of γTuRC. The fraction was desalted, concentrated ~20-fold, and then tested in the complementation assay. We found that this salt-treated 30% Asp also complemented the γTuRC-depleted extract to form a functional centrosome (Fig. 7). Since we know that the γTuRC in this pellet is dissociated by the high-salt incubation (see above), this complementation assay allows us to study the effect of selective immunodepletion of Xgrips on centrosome assembly.

Immunodepletion of Xgrip109 Abolishes the Complementing Activity

To study the role of Xgrip109 in γ-tubulin recruitment and centrosome formation, we wanted to deplete the Xgrip109

from the salt-dissociated γTuRC present in the resuspended Asp, and ask whether the remaining γTuRC components still function in the complementation assay described above. Xgrip109 was immunodepleted from the pellet resuspended in high-salt buffers (Fig. 8 *A,* lanes *4–8*). Control reactions were immunodepleted with random IgG. The two antibody-depleted samples were desalted into low-salt buffers and then concentrated ~20-fold (Fig. 8 *A,* lanes *5–8* for the extent of immunodepletion). We found that immunodepleting Xgrip109 abolished the complementing activity present in the resuspended pellet (Fig. 8, *B* and *C*), whereas the random IgG control retained the complementing activity (Fig. 8, *B* and *C*). Although immunodepleting Xgrip109 removed a fraction of γ tubulin (refer to Fig. 5 *B*, lane *2*), we estimated that more than half of the total amount of γ tubulin remained (Fig. 8 *A*, lanes *4–8*). Interestingly, this remaining γ tubulin did not appear to be recruited to the centrosome (Fig. 8 *C*, γ-tubulin staining of the centrosomes). These results suggest that Xgrip109 is necessary for the recruitment of γ tubulin to the centrosome, and for the formation of a functional centrosome.

Discussion

Xgrip109 Is a Conserved γ Tubulin–Interacting Protein

The γTuRC consists of approximately eight polypeptides (Xgrips) in addition to γ tubulin. In an effort to further understand the assembly and function of the γTuRC, we have begun to characterize the γTuRC components. We cloned and sequenced one of the Xgrips, Xgrip109. Sequence analysis revealed that Xgrip109 is homologous to the yeast γ tubulin–interacting protein Spc98 (Geissler et al., 1996) (refer to Fig. 1). Like Spc98, Xgrip109 also interacts with γ tubulin (refer to Fig. 5). This suggests that Xgrip109 and Spc98 may have similar cellular functions. In *Xenopus,* Xgrip109 is a component of the γTuRC. The *S. cerevisiae* Spc98 is also a component of a γ-tubulin–containing complex, although the yeast γ-tubulin complex has a much smaller S value than that of the γTuRC (Geissler et al., 1996; Knop et al., 1997). It will be interesting to study whether the smaller yeast γ-tubulin complex, like the γTuRC, can nucleate microtubules in vitro.

EST database searches shows that Xgrip109 is highly conserved among humans, mice, fish, rice, and flies. The Xgrip109 EST homologues in vertebrates can be divided into two groups that share sequence identities with a similar region of Xgrip109 (refer to Fig. 2). One group of ESTs shares over 85% amino acid identity with Xgrip109 and with each other in the 140-amino acid overlapping sequences. The other group of ESTs shares over 30% amino acid identity with Xgrip109, and over 85% amino acid identity with each other in the 80-amino acid overlapping sequences (refer to Fig. 2). Approximately 30% of amino acid identity is shared between the two groups of ESTs. This suggests that Xgrip109 is highly conserved in vertebrates and that it belongs to one of the two families of related proteins. We have recently identified two related γ-tubulin–interacting proteins that are components of *Drosophila* γTuRC and found that each shares sequence homology with one of the two families of proteins (our unpublished data). We suggest that there are two related families of conserved γ-tubulin interacting proteins that participate in the formation of the γTuRC.

Xgrip109, γ Tubulin, and γTuRC Assembly

We found that the salt-dissociated γTuRC present in a resuspended 30% Asp can reassemble after desalting (refer to Fig. 6). The reassembly was judged by the shifting in the S value of γ tubulin and Xgrip109 to that of the γTuRC in our experiments. Because only ~50% of the total γTuRC can reassemble, we did not attempt to purify the reassembled γTuRC due to the limited amount of starting material. Therefore, we did not carry out structural analysis to compare the reassembled γTuRC with that of the endogenous γTuRC. However, we believe that the reassembled γTuRC is at least similar to the endogenous γTuRC for two reasons. First, immunoprecipitation using anti-Xgrip109 and γ-tubulin antibodies showed that the reassembled γTuRC contained the same set of proteins as that of the endogenous γTuRC (data not shown). Second, the reassembled γTuRC can functionally replace the endogenous γTuRC in the centrosome formation assay (see below).

Using the γTuRC reformation assay, we found that removing Xgrip109 and a small fraction of γ tubulin before desalting blocks the ability of the remaining γ tubulin to assemble into a γTuRC-sized complex after salt removal. In fact, the remaining γ tubulin has a similar S value before and after desalting. This suggests that Xgrip109 is required for γ tubulin to assemble into larger complexes.

We only looked at γTuRC dissociation and reassembly using 30% ammonium sulfate–precipitated γTuRC. It will be interesting to test whether the purified γTuRC can also reassemble after dissociation with high salt, or whether accessory factors present in the 30% Asp fraction are required. Unfortunately, since we can only purify a few micrograms of γTuRC, this type of experiment is currently impractical. Nevertheless, the fact that the dissociated γTuRC can be reassembled offers a useful assay to study how γTuRC is assembled from its subunits.

Xgrip109 Is a Centrosome Component

γ-Tubulin–containing rings that have similar dimensions to that of the purified γTuRC were found in the PCM of purified centrosomes (Moritz et al., 1995*a*,*b*). One important question is whether the γTuRC or only γ tubulin in the γTuRC is recruited to the PCM to act as a microtubule nucleator. Because the purified γTuRC can nucleate microtubules in vitro, we propose that the γTuRC-like rings in the centrosome contain at least some of the Xgrips present in the γTuRC (Zheng et al., 1995). The finding that Xgrip109 is a centrosomal protein that interacts directly with γ tubulin suggests that Xgrip109 is likely a component of the centrosomal rings.

The Complementation Assay for Centrosome Assembly

To study how each of the Xgrips is involved in the assembly of a functional centrosome, we developed a complementation assay for centrosome formation. In this assay the centrosome formation activity depends on the combination of two fractions: a γTuRC-depleted extract and a 30% Asp fraction that contains the γTuRC. Since the

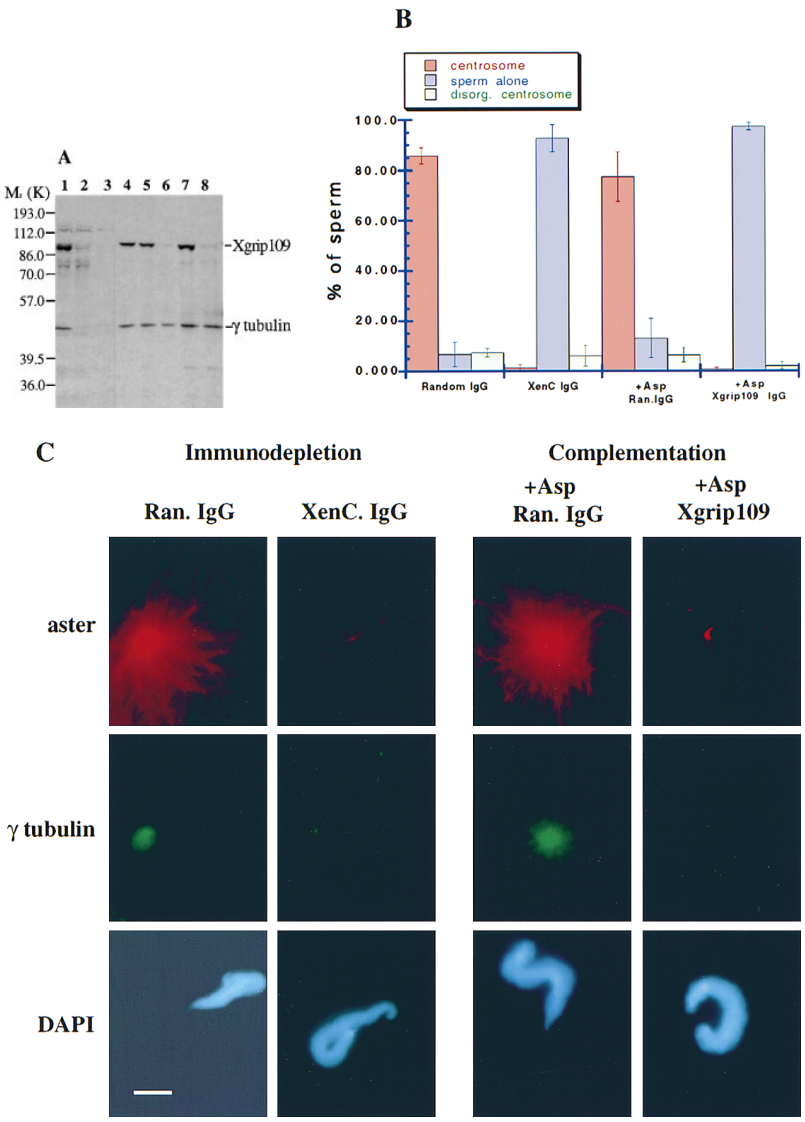

Figure 8. Xgrip109 is essential for the formation of a functional centrosome. (*A*) Western analysis of the immunodepleted extract, 30% Asp, and 30% ammonium sulfate supernatant probed with XenC and 109-2. Lanes *1* and *2*: clarified extract immunodepleted with either random IgG (lane *1*) or XenC (lane *2*). Judging by the absence of γ tubulin and Xgrip109, XenC depleted the γTuRC (lane *2*). Lane *3*, 30% ammonium sulfate supernatant that does not contain detectable γTuRC as expected. Lane *4*, 30% Asp resuspended in Hepes 1 M. Lanes *5* and *6,* 30% Asp resuspended in Hepes 1 M and then immunodepleted using either random IgG (lane *5*) or 109-2 (lane *6*). Lanes *7* and *8* are the same as lanes *5* and *6*, respectively, except that the proteins were desalted into Hepes 100, and concentrated ~20-fold. Lanes *5–8* show that immunodepletion of Xgrip109 in 1 M KCl removed only a fraction of γ tubulin (compare γ-tubulin signals in lanes *5* and *6*), whereas Xgrip109 is completely depleted. (*B*) Quantitation of the complementation assay. *Red columns*, percentages of sperm with microtubule asters nucleated from the assembled centrosomes; *blue columns*, percentages of sperm without microtubule asters; *white columns*, percentages of sperm with assembled centrosomes that nucleated only a few disorganized microtubules; *Ran. IgG*, centrosome formation assays carried out with clarified extracts that were immunodepleted with random IgG. Over 80% of the sperm centrioles assembled into centrosomes. *XenC. IgG*, centrosome formation assays carried out with clarified extracts that were immunodepleted of γTuRC using XenC IgG. The centrosome assembly activity was abolished. *+Asp Ran. IgG*, 30% Asp that was resuspended in Hepes 1 M and immunodepleted with random IgG complemented the γTuRC-depleted extract to assemble centrosomes. *+Asp Xgrip109*, 30% Asp that was resuspended in Hepes 1M and immunodepleted of Xgrip109 did not complement the γTuRC-depleted extract to assemble centrosomes. (*C*) Representative sperm nuclei with or without a microtubule aster from the assays in *B* are shown. The microtubules were labeled by the addition of a small amount of rhodamine-tubulin in the assays. γ Tubulin was detected using an anti–γ-tubulin monoclonal antibody GTU-88 (Sigma Chemical Co.) and a fluorescein-conjugated goat anti–mouse secondary antibody. γ Tubulin is not recruited to the centrosome in the absence of Xgrip109. The sperm DNA was stained by DAPI. Bar, 10 μm.

γTuRC in the pellet can be dissociated with high salt and reassembled by removing the salt, we can remove specific subunit(s) by immunodepletion in high salt, remove the salt, and then study whether the remaining components reform a complex that can function in centrosome assembly. We believe that this type of assay will also be useful in studying other centrosomal components.

We know that the γTuRC in the Asp is the key activity that complements the γTuRC-depleted extract, because depleting Xgrip109 (Fig. 8) or γTuRC (our unpublished observation) abolishes the activity. An obvious question is whether the purified γTuRC is sufficient for the complementation. To address this question in a meaningful way, we are currently improving our purification methods to achieve sufficiently high γTuRC concentrations for the complementation assay.

γ Tubulin, Xgrip109, γTuRC, and Centrosome Assembly

This study and previous work by Felix et al. (1994) strongly suggest that γTuRC is essential for centrosome assembly. An interesting question is how the γTuRC is recognized by the centrosome assembly machinery. One model is that the intact γTuRC is recognized by a recruiting factor(s) that assembles the whole γTuRC to the centrosome (Fig. 9 *A*). In this case, the observed centrosomal γ-tubulin–containing rings (Moritz et al., 1995*a*,*b*) would be similar to the γTuRC. In an alternative model, γTuRC is merely a cytoplasmic storage form of the centrosomal ring components. Upon centrosome assembly, γTuRC is disassembled and only γ tubulin, and possibly some of the Xgrips, such as Xgrip109, are recognized and recruited to the centrosome to form the centrosomal ring (Fig. 9 *B*).

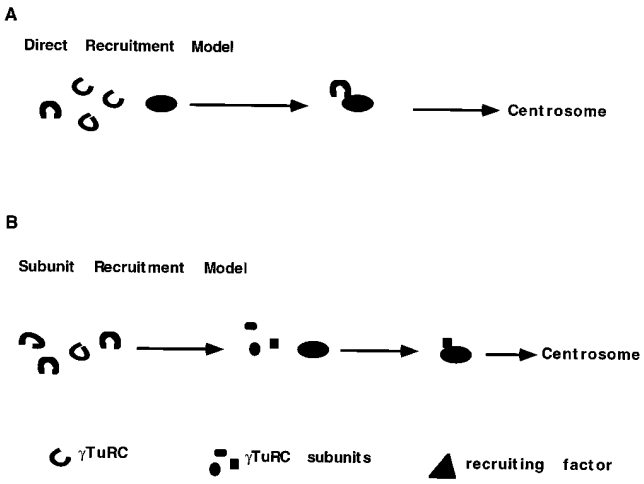

Figure 9. Recruitment models. (*A*) Direct recruitment model. γTuRC directly binds to a hypothetical recruitment factor(s) and assembles to the centrosome to act as a microtubule nucleator. (*B*) Subunit recruitment model. γTuRC is first disassembled to subunits. Only some subunits are recruited to the centrosome by the recruitment factor.

Although we cannot yet differentiate between these two models, our findings suggest that the centrosome assembly machinery cannot recognize and recruit γ tubulin unless it is in the γTuRC or associated with Xgrip109. When all of the Xgrip109 and a small amount of γ tubulin was removed, the remaining γ tubulin did not reassemble into the γTuRC and was not recruited to the centrosome (refer to Figs. 7 and 8). Since only ~50% of the total γ tubulin remained upon Xgrip 109 depletion, it is formally possible that the failure of recruiting this remaining γ tubulin is merely due to insufficient γ-tubulin concentration. However, we believe that this is an unlikely possibility for the following reasons. First, in our centrosome formation assays, the extract was always diluted to 60% of the original concentration (refer to Materials and Methods) and the centrosome formation and γTuRC recruiting were not significantly affected. Furthermore, Felix et al. (1994) showed that the extract can be diluted threefold without drastically affecting the centrosome formation activity. These observations suggest that the recruitment of γ tubulin to the centrosome should not be affected when the amount of γ tubulin is only diluted onefold. We are currently testing the role of Xgrip109 in γ-tubulin recruitment.

In conclusion, we have initiated molecular characterizations of the γTuRC components. With the assays presented in this paper, we hope to determine the role of each of the γTuRC subunits in centrosome formation and microtubule nucleation.

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