

The Role of Xgrip210 in γ -Tubulin Ring Complex Assembly and Centrosome Recruitment

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Abstract. The γ -tubulin ring complex (γ TuRC), purified from the cytoplasm of vertebrate and invertebrate cells, is a microtubule nucleator in vitro. Structural studies have shown that γ TuRC is a structure shaped like a lock-washer and topped with a cap. Microtubules are thought to nucleate from the uncapped side of the γ TuRC. Consequently, the cap structure of the γ TuRC is distal to the base of the microtubules, giving the end of the microtubule the shape of a pointed cap. Here, we report the cloning and characterization of a new subunit of *Xenopus* γ TuRC, Xgrip210. We show that Xgrip210 is a conserved centrosomal protein that is essential for the formation of γ TuRC. Using immunogold

labeling, we found that Xgrip210 is localized to the ends of microtubules nucleated by the γ TuRC and that its localization is more distal, toward the tip of the γ TuRC-cap structure, than that of γ -tubulin. Immunodepletion of Xgrip210 blocks not only the assembly of the γ TuRC, but also the recruitment of γ -tubulin and its interacting protein, Xgrip109, to the centrosome. These results suggest that Xgrip210 is a component of the γ TuRC cap structure that is required for the assembly of the γ TuRC.

Key words: Xgrip210 • centrosome • microtubule nucleation • γ -tubulin ring complex • *Xenopus*

Introduction

The microtubule (MT)¹ cytoskeleton is assembled from highly dynamic polymers of α - and β -tubulin heterodimers. It is found in all eukaryotic cells and is vital for many cellular functions such as intracellular organization, membrane trafficking, and cell division. The dynamic nature (Mitchison and Kirschner, 1984a) and spatial control of MT assembly allows this cytoskeleton to change its shape and mass efficiently in response to cell cycle and developmental cues (Desai and Mitchison, 1998).

Spatial and temporal control of MT nucleation in different organisms is carried out by specialized organelles collectively called the MT organizing centers (MTOC). The major MTOC found in animal cells is the centrosome, which was first observed >100 yr ago (Wilson, 1925). The centrosome, which consists of a pair of centrioles and a pericentriolar material that contains the MT nucleating ac-

tivity (Gould and Borisy, 1977), is found juxtaposed to the nucleus of most fibroblastic cells. The centrosome not only nucleates MTs more efficiently than spontaneous MT nucleation (Mitchison and Kirschner, 1984b), but also participates in organizing the interphase MT arrays that are critical for intracellular trafficking (Kellogg et al., 1994). Progression through the cell cycle into mitosis is accompanied by many changes in the MT cytoskeleton including increased MT nucleation by the centrosome (Kellogg et al., 1994). The increase in MT nucleation is believed to be important for spindle assembly and organization (Heald et al., 1997; Hyman and Karsenti, 1998). Thus, to understand how the MT cytoskeleton is regulated throughout the cell cycle, it is critical to understand how the centrosome promotes MT nucleation.

The discovery of γ -tubulin (Oakley, 1992) initiated a new era of studying MT nucleation by the centrosome (Wiese and Zheng, 1999). Identified as a suppressor of a β -tubulin mutation in the filamentous fungus, *Aspergillus nidulans* (Oakley and Oakley, 1989), γ -tubulin is a highly conserved protein localized to all MT nucleating sites examined thus far (Wiese and Zheng, 1999). Genetic studies in *A. nidulans* (Oakley et al., 1990), *Saccharomyces cerevisiae* (Sobel and Snyder, 1995; Marschall et al., 1996; Spang et al., 1996), *Schizosaccharomyces pombe* (Horio et al., 1991), and *Drosophila melanogaster* (Sunkel et al., 1995;

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¹Abbreviations used in this paper: CSF, cytosolic factor; Dgrip, *Drosophila* gamma ring protein; γ TuRC, γ -tubulin ring complex; γ TuSC, γ -tubulin small complex; MT, microtubule; Xgrip, *Xenopus* gamma ring protein.

Tavosanis et al., 1997) suggest that γ -tubulin is involved in MT nucleation. Biochemical studies of γ -tubulin in *Xenopus* eggs and *Drosophila* embryos led to the purification of a 2-MD γ -tubulin ring complex (γ TuRC) that can nucleate MT assembly in vitro (Zheng et al., 1995; Oegema et al., 1999). Most γ -tubulin in animal cells appears to exist as γ TuRC (Wiese and Zheng, 1999), and several studies indicated that γ TuRC is recruited to the centrosome to function as a MT nucleator (Felix et al., 1994; Martin et al., 1998; Moritz et al., 1998; Schnackenberg et al., 1998). For example, electron tomographic reconstruction of isolated *Drosophila* and *Spisula* centrosomes revealed that the pericentriolar material of these centrosomes contains hundreds of γ TuRC-like structures (Moritz et al., 1995; Schnackenberg et al., 1998). In addition, in vitro centrosome reconstitution assays reveal that γ TuRC is essential (but not solely sufficient) for the formation of a functional centrosome (Martin et al., 1998; Moritz et al., 1998).

Analysis of γ -tubulin complexes purified from *Drosophila* embryos has offered important insights into the organization of the γ TuRC. The *Drosophila* γ TuRC can be dissociated into a smaller γ -tubulin-containing complex, the γ -tubulin small complex (γ TuSC), that is a tetramer of two γ -tubulins and one each of the *Drosophila* gamma ring protein (Dgrip) 91 and Dgrip84. Stoichiometric analyses have suggested that each γ TuRC consists of approximately six γ TuSCs that make up the ring wall of the γ TuRC as revealed by cryoelectron microscopy (Oegema et al., 1999; Wiese and Zheng, 1999). Recent electron tomographic reconstruction of the *Drosophila* γ TuRC shows that it consists of a lock-washer-shaped ring that is covered with a cap on one face (Moritz et al., 2000). When viewed from the side, the γ TuRC ring consists of repeated, hairpin-shaped subunits that were proposed to correspond to γ TuSCs (Moritz et al., 2000). The cap structure possibly consists of the non- γ TuSC subunits named Dgrips163, 128, and 75s (Keating and Borisy, 2000; Moritz et al., 2000). This structural organization suggests that the cap structure may be important for the assembly of multiple γ TuSCs into one γ TuRC.

Xenopus γ TuRC is similar to *Drosophila* γ TuRC in its subunit composition, structure, and function (Zheng et al., 1995; Oegema et al., 1999; Wiese and Zheng, 1999). Here we report the characterization of a *Xenopus* gamma ring protein (Xgrip) 210 as a potential subunit for the cap structure that is required for the assembly of the γ TuRC and its recruitment to the centrosome.

Materials and Methods

Buffers

Hepes 100 (mM): 50 Hepes, pH 8, 1 MgCl₂, 1 EGTA, and 100 KCl. Hepes 1M: the same as Hepes 100 except that the concentration of KCl is 1 M instead of 100 mM. Cytostatic factor (CSF)-XB (mM): 10 potassium Hepes, pH 7.7, 100 KCl, 2 MgCl₂, 0.1 CaCl₂, 50 sucrose, and 5 EGTA. BRB80 (mM): 80 potassium Pipes, pH 6.8, 1 MgCl₂, 1 EGTA. MT-stabilizing buffer (mM): 100 Pipes, pH 6.9, 5 EGTA, 10 MgCl₂, 10 μ g/ml Taxol.

Cloning of Xgrip210

Mouse polyclonal ascites against Xgrip210 were generated as described (Martin et al., 1998). The antibodies were used to screen a λ ZAP cDNA library of *Xenopus* oocytes (Stratagene) as described (Sambrook et al., 1989), with modifications (Hirano and Mitchison, 1994). To obtain the missing 5'

end, we carried out 5' rapid amplification of cDNA ends using a 5'/3' RACE Kit (Boehringer). Three partially overlapping primers (gsp1, GGT GAG AAG AGT CAA TGA TGC; gsp2, TGC AGG AGT TGA TAA AAC ACA; and gsp3, CTG CAG ATA TTT CCT AAG GCC) corresponding to the 5' region of the longest cDNA clone were used in the RACE reaction. The longest RACE product contained 234 amino acids that were missing from the original clone. We subcloned the RACE product into the 5' end of the original cDNA construct and used the resulting construct as well as the original cDNA construct to translate proteins in rabbit reticulocyte lysates (Promega). The translated proteins were fractionated on a 7% SDS polyacrylamide gel together with *Xenopus* egg extract and analyzed by Western blot, probing with antibodies against Xgrip210.

Antibodies and Immunoblotting

To raise rabbit polyclonal antibodies against Xgrip210, a fusion protein was made between glutathione S-transferase (GST) and a fragment of Xgrip210 corresponding to amino acids 874–1097. This fusion protein was purified as described (Martin et al., 1998), and used to generate rabbit polyclonal antibodies. After depleting anti-GST antibodies from the antisera, the antibodies were affinity purified against the fusion protein. The antibodies against Xgrip109 and *Xenopus* γ -tubulin used here were described previously (Martin et al., 1998). The monoclonal antibody against γ -tubulin was commercially available (GTU-88; Sigma-Aldrich).

Immunoblotting was carried out using affinity-purified antibodies at a concentration of \sim 1 μ g/ml using either an ECL detection system (Amersham Pharmacia Biotech) or an alkaline phosphatase detection system (Promega).

Xenopus Egg Extract, *Xenopus* Demembrated Sperm, Sucrose Gradient Sedimentation, Immunoprecipitation, and Other Assays

Crude or clarified *Xenopus* egg extracts and demembrated *Xenopus* sperm were prepared as described (Martin et al., 1998). The preparation of a 30% ammonium sulfate pellet, immunodepletion, and all of the assays used in this manuscript were described previously (Martin et al., 1998). Sucrose gradient sedimentation was carried out on either 5–40% or 10–50% continuous gradients, as described (Martin et al., 1998) with minor modifications in making the 5–40% sucrose gradient. To make the 5–40% continuous sucrose gradient, step gradients were made with equal volumes of 5, 13.75, 22.5, 31.25, and 40% sucrose in Hepes 100 and allowed to diffuse into continuous gradients. This modification allowed better resolution of the Xgrip210, Xgrip109, and γ -tubulin-containing protein complexes than the gradients used previously. Fractions were collected manually from top to bottom with a cut-off pipette tip.

Cell Culture and Immunofluorescence Staining

The *Xenopus* cell line, XLK-WG, derived from a primary culture of *Xenopus* kidney cells (provided by Drs. Z. Wu and J.G. Gall, Carnegie Institution of Washington, Washington, DC) was used for immunofluorescence staining as described (Martin et al., 1998). Xgrip210 was localized to the sperm centrosome according to a previously described method (Martin et al., 1998). When double labeling γ -tubulin and Xgrips 109 or 210 was carried out, secondary antibodies against mouse or rabbit (Molecular Probes, Inc.) conjugated with Alexa Fluor 488 or 594, respectively, were used.

Photomicrographs were obtained using a cooled CCD camera (Princeton Scientific Instruments, Inc.) on a Nikon E800 microscope. Images were processed digitally using Adobe Photoshop (Adobe Systems Inc.).

Immunogold Labeling and Electron Microscopy

Recombinant dynamitin was added to *Xenopus* CSF egg extract at a final concentration of 0.5 mg/ml. MT nucleation in the extract was induced by DMSO (5% vol/vol final) at room temperature for 30 s, at which time the extract was diluted 50-fold with MT-stabilizing buffer plus 0.1% Triton X-100. Diluted extract was immediately loaded onto a 4-ml cushion of MT-stabilizing buffer plus 10% glycerol in a 15-ml Corex tube. At the bottom of the tube was a plastic support holding a 12-mm round coverslip with Formvar-coated EM grids attached. The Corex tubes were centrifuged in a Sorvall HB-4 swinging bucket rotor at 6,000 g for 30 min at 25°C. The coverslips with attached grids were removed and rinsed with MT-stabilizing buffer and individual grids were immunostained with Xgrip210 antibodies conjugated to 10-nm gold particles as described (Keating and Borisy, 2000). Conjugation of Xgrip210 antibodies to 10-nm gold particles was performed using a method (Hughes and Beesley, 1998) modified as previously

MATVAECNHSVLDLVTMKMATLVADETKPKKQTAALVAKKRKSIYESSLLHSETMSSQDKL	60	Dgrip163	DQPRGKE . CSLPRVVFQYQRSHSDV . INSNEHPT . . TQA EFNRRQ	773	Dgrip163
MLSF . . . NLRICGMSSEADLR . . . EELVEYLEQNSGIQISDLHVALELVE . . . LSGTGPP	52	Xgrip210	DT . KDKESLALKSDLQESSTGSEYDFKTLIKPIACTQVSGHQHIKIGEYSSNVQPARPRW	930	Xgrip210
EMLHQVTEVAKNPRDMMLLRPQNLEGGDRESEITQDQDEENQSEVVIKRIKAGPLQ	120	Dgrip163	. . HGLSSDQ FQQCHATVHLQESDLLKTAASTSSGLHAEIPQ . DINANL	818	Dgrip163
QLLPP . KRDIYFKNNKIVGRNVIKQ . . . GYDIYDVQVFE . . . ADLGTTVAYQLEISTT .	103	Xgrip210	STHGSSSDSNIKIGNYVDPINVHQPKHSQHGSSSDSNINISDHMSDVEPRLRNLHCHI	990	Xgrip210
LIEIEIVEEA . . . LHPKAFDPINQM . NRALKPLQLQPYSDIL . . TTSNLNI . . NFPP	170	Dgrip163	NCPDTK . . ELTELQRNRKRNEHHGDFFSFN . . . STEDEHTRRLR . . . SLLNSNTE	865	Dgrip163
IQRITLQIMEAAPTGLPALSFQNDLSSDKFEKETRGSILFAGLHVSRNTDMDIKLDMPP	163	Xgrip210	STGHIKVEYASDVEPSTPRHSVHGASQGNKIKIGENVSDVLKSRPWNHGHVSDANIK	1050	Xgrip210
EKLIKPIIPTVIRMGLESITRMRNE MEKFMHP P	205	Dgrip163	RGRNRLRM ETEFGLGYKNTDIET . . . KATP CLPL . . . EI	899	Dgrip163
. . . VPENADLSGLAIKVPQSIDQSEDEGFSASNMTPDSQSEPSMTPIDVWEAVLTYGP	220	Xgrip210	IGENTTEIAPLRPWNHGHASQSHIKIGELVSDIEFSQPRRTFFGHPSQSSIPIGDQV	1110	Xgrip210
PRLPVLAKKVVAPLEDERPFVAQPGPEKHVSDKLLTNELKILRNKLV . . LNYLNEATL	263	Dgrip163	DK LHVEIP LAVLTPMSTSDVDF	922	Dgrip163
SKRRCWERICGPPGKREPEYVTEAGREAFDKLYLHEGGQLSATTLPQQLVLEETDL	280	Xgrip210	EKYAQKSESEVHSSNSTIQHLLYSNIPDKNKDTGGTLTDSFVVPDQGNSSNDTEKRSST	1270	Xgrip210
IDHLKKAACGIESLTFFISSADKLELRVPTTLPTMLPEVLADFANHFIRAGCAFLRLS	323	Dgrip163 GDPS RELPEIADT ANNN VSGSIE	947	Dgrip163
VKAVLVNLIGVVSSTFSYQALQ . SFAVKQGVYISGTSPPNVSSLLTQVAEYGYTYRSL	339	Xgrip210	LEQRVQAADSVDCGEASPNTAQSLPCMSDLDLGTNGEENGVNDHTWEKQOYELKGLAE	1230	Xgrip210
SRTKWNNIAEMRLERFLNRAMRETIDYLSSTRQFLLSLQADS LFQLLNNTST	376	Dgrip163	SL SPELPK PVLAIAKPTSLLP . KLDGFMKDERETEQRDKVSTLPEACNPF	997	Dgrip163
HFSLLTVLDSHNSGLVQAFSTGLRKYLYQYRACVLSSTPASLTLTISFLRKLGRQLR	399	Xgrip210	KYCLEKYQSYELMSPVPLHLHY . . SNVMPNRFSTQDSDIKSATDETTVQLIELLSLV	1288	Xgrip210
AIQLLQDRMFENEPRLNLTNGI . TGSFLLSITWL . AIDTCGNKDFLHLLIYFLRCISR	434	Dgrip163	MARRCQLSVMAPLNAYGLLRNEVL . RIFQEQRIYEHFRKRLNYFFLVDCGQATLNE	1056	Dgrip163
YLAELECCIGTLVTSATR . GISTAFPTGKLLSYLYKEALENSSENENY . FVLLSLKTSCE	457	Xgrip210	L MKYSVTPAMVSHVLYVKAIVDYFVVELKMERHFEAMRHFLMEDGEFAQSLSDM	1344	Xgrip210
<u>NYFIQLQRWIYHGEDESVEINFIISRLCNTSPSFTNQCSKEFFDKSY . QVDNEA . . IPE</u>	490	Dgrip163	<u>TLGRIRAGVGRPSLSQKGLDILTILNALAACSADETTVSENLTNCTTIPDILNFLSVEA</u>	1116	Dgrip163
<u>PYTRFIYDWMVSGYFRDVCGEFMIQ . . . VNEDYLGDFDKRWTHGYVLSIKEVEDCVPV</u>	513	Xgrip210	<u>LFELKSGQTPSELLNPLVLSILNKALQYSLHGDSSLASLNTFALKYLFVFTPTAPDA</u>	1404	Xgrip210
FLVGCEEEILQCGKYNLVRAYNAQHPVF . . DVQYPIVVCLEQQKMKMRNLADKYAT	548	Dgrip163	<u>TSMLKLNCKIDWPLNHWISSETISKYQIFGYLLKLRHVSFLVDGTYEYLQMGKLLGPE</u>	1176	Dgrip163
FLKHVANEIYICGKTINLLKLCPRHYICWSDIPVRIISVTFSLLEELKEMEKDCA . VYVA	572	Xgrip210	<u>LSCLELKYKVDWPLNIVITDTCMNKYSRIFSLQLKHMVWTLRVDVWFHLKRTA . LVNQ</u>	1462	Xgrip210
IYKRFGW . C SMQS IFEDRMATKRVFANLWVKTQAHLDWAQKQRE	593	Dgrip163	<u>LRTCTHFRHMGMHRKMSHFMTSFOHLVAKALLSTWKSFEELCTADSIFALYKQHVAY</u>	1236	Dgrip163
RMERLARHCSISKEQKALQTEIARQELIIQARETTEKVFETFKORLAEKL . SLDTKKRE	631	Xgrip210	<u>ASNSVQYRQLQLYRHEMQRHVVKYIQGYIANQILHVTWCFFRNKLSAVSNLEEYKTHADY</u>	1522	Xgrip210
LQIKANAQ . KKLQNDQNAEQE RIQ . . QNHLE . KRRQDIVNEL	632	Dgrip163	<u>LKRVAFLALLNRRSAKVKETIDNIVLILRCKVQISQSFVVDQNYF . VHPFRKLRQE</u>	1295	Dgrip163
LFQKLKQYKEQERRLTTKQEEADDDFSYAREIRDREKRLKALEEELKTRQELIEHY	691	Xgrip210	<u>LNKALFRGLLTKAAAPLMIITHSIFSLIKFLRLQISQSWICDTGKMAVHPNFMGLMQS</u>	1582	Xgrip210
A . PORECERMEDKLL EREKQEL QKVVQLA LSSSPD .	669	Dgrip163	<u>EAEFEKFLQYLYLGNKAAASGVQEEIGDLICINFNHYVYKSGNHTGSSMEDKCC</u>	1351	Dgrip163
SRLSEEARTRKEQALWKLQRHKLLETTRILKFLFEEQKRNQDLVANFPVDICEENLGVLPDG	751	Xgrip210	<u>YNTFKYSDFLFEVVSKLVRGYQPHLEDFLLRINFNYSYKQS</u>	1625	Xgrip210
. RSTVSDLSFASCIEE PD CLAESSSDKDDAN . . TENS	703	Dgrip163			
EISHQONTNDAGLNIENKSVPEQHALHNNDEVYTAQNCISKSESICVDVTLPTENV	811	Xgrip210			
EDEATK QLDKTLNVP LEQPEAEYQ ADVDV	732	Dgrip163			
HSQTSNASVGLVPSFSDSNLCTPDVDVIDIFLPTLPSENQEVAVVQSLVDDALISIGDLNT	871	Xgrip210			

Figure 1. Xgrip210 defines a new family of grips of γ TuRC. Sequence comparison of Xgrip210 and Dgrip163. The two sequences share over 20% amino acid identity. Double dots indicate identical amino acids, and single dot denotes the conserved changes. The sequences corresponding to grip motifs 1 and 2 found in the amino and carboxyl half of the proteins, respectively, are underlined.

described (Keating and Borisy, 2000). Grids were examined with a Philips 300 TEM at 80 kV and photographed at 64,000 \times . Negatives were scanned at 1,200 dpi and analyzed using Adobe Photoshop. We chose microtubules that have only one gold particle at the capped ends to compare the Xgrip210 localization with γ -tubulin localization that we determined previously using the same conditions (Keating and Borisy, 2000).

Results

Molecular Characterization of Xgrip210

To identify its components, *Xenopus* γ TuRC was purified and used for protein sequencing and mouse polyclonal antibody production (Martin et al., 1998). We successfully generated mouse polyclonal antibody against Xgrip210, the largest Xgrip found in the *Xenopus* γ TuRC. We referred to Xgrip210 as Xgrip195 in previous publications (Zheng et al., 1995; Martin et al., 1998; Oegema et al., 1999), but propose to change its name to Xgrip210 to more accurately reflect its apparent molecular mass of 210 kD.

Using the mouse polyclonal antibody, we cloned a number of cDNAs from a *Xenopus* oocyte cDNA expression library. The longest cDNA was joined with the additional 5' coding sequences of Xgrip210 obtained by 5' rapid amplification of cDNA ends. When translated in vitro, the joint cDNA clone produced a protein similar in size to Xgrip210 found in the *Xenopus* egg extract (data not shown). This suggests that we have the complete coding sequence for Xgrip210. The cDNA sequence (accession

No. AJ291606) predicts a protein of 1,625 amino acids with a pI of 5.6.

The COOH-terminal \sim 1,000 amino acids of Xgrip210 has sequence homology (>40% identity) to a partial human expressed sequence tag (No. AL022328). Therefore, we suggest that this human protein is the putative homologue of Xgrip210, which we name Hgrip210. Xgrip210 also shares homology with Dgrip163, the largest grip found in *Drosophila* γ TuRC (Fig. 1). Both Xgrip210 and Dgrip163 contain two sequence motifs (grip motifs 1 and 2, see the underlined sequences in Fig. 1) that are conserved in all grips identified thus far (Gunawardane et al., 2000).

Xgrip210 Is a Component of the *Xenopus* γ TuRC and Is Localized to the Centrosomes

To further characterize Xgrip210, we generated rabbit polyclonal antibodies against the protein. The affinity-purified antibodies recognize a single protein in *Xenopus* egg extracts with the expected size for Xgrip210 (Fig. 2 A). When antibodies against Xgrip210, Xgrip109, or *Xenopus* γ -tubulin were used to immunoprecipitate the corresponding proteins from *Xenopus* egg extracts, all three antibodies immunoprecipitated the same set of *Xenopus* γ TuRC proteins, showing that Xgrip210 is a component of the γ TuRC (Fig. 2 B). In addition, Xgrip210 and γ -tubulin cosedimented on sucrose gradients (Fig. 2 C), further demonstrating that Xgrip210 is a subunit of the

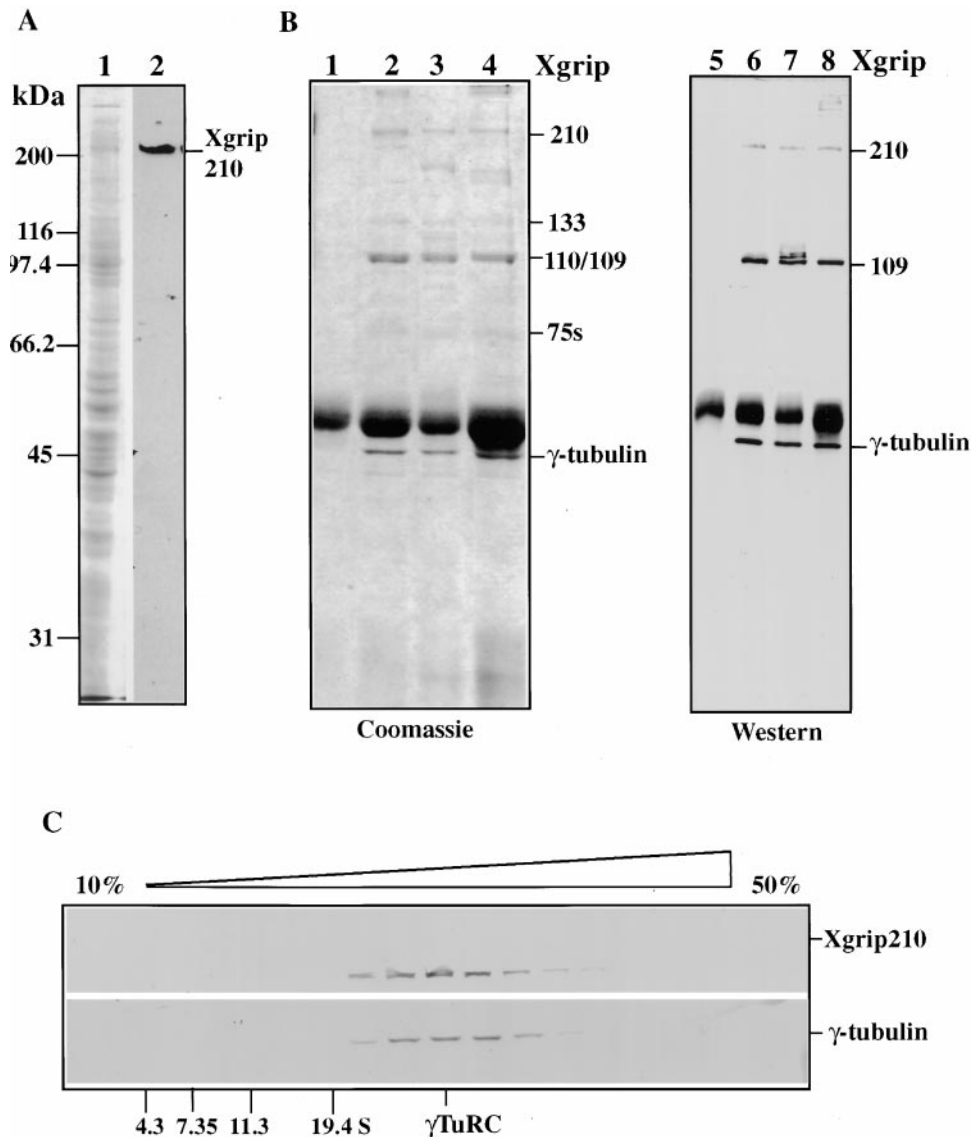


Figure 2. Xgrip210 is a component of *Xenopus* γ TuRC. (A) Crude *Xenopus* egg extracts were analyzed by Coomassie blue staining (1) or by Western blot, probing with anti-Xgrip210 antibodies (2). The antibodies specifically recognized a protein of the expected size for Xgrip210. (B) Random rabbit IgG (1 and 5) and antibodies against Xgrips210 (2 and 6) or 109 (3 and 7) or γ -tubulin (4 and 8) were used to immunoprecipitate the respective proteins from *Xenopus* egg extracts. The immunoprecipitates were analyzed by SDS-PAGE followed by Coomassie blue staining (1–4) or Western blot, probing with each of these antibodies (5–8). All three antibodies immunoprecipitated the same γ TuRC subunits. Although the Xgrip210 antibody specifically recognized Xgrip210 in the egg extracts (A, 2), the antibody recognized several high molecular weight proteins in the Xgrip210 immunoprecipitate (B, 8). The nature of these cross-reactive proteins is currently unknown. (C) Clarified *Xenopus* egg extract or protein standards with S values of 4.3 S (bovine serum albumin), 7.35 S (rabbit muscle aldolase), 11.3 S (bovine liver catalase), and 19.4 S (porcine thyroglobulin) were run on parallel 10–50% sucrose gradients. The fractions were analyzed by Western blot, probing for γ -tubulin or Xgrip210 or by Coomassie blue staining for the protein standards.

γ TuRC. Using immunofluorescence microscopy, we localized Xgrip210 to the centrosomes of *Xenopus* tissue culture cells and *Xenopus* sperm incubated in egg extracts (Fig. 3). Therefore, like its homologue Dgrip163 (Gunawardane et al., 2000), Xgrip210 is a centrosomal protein.

Xgrip210 Localizes to the Distal Portion of the γ TuRC-capped MT End

Structural studies showed that the minus ends of MTs nucleated from the γ TuRC have a distinct cap (Keating and Borisy, 2000; Moritz et al., 2000; Wiese and Zheng, 2000). The distal portion of this cap is thought to correspond to the cap structure of the γ TuRC (Moritz et al., 2000). We have previously localized γ -tubulin and Xgrip109 to the ends of MT capped by γ TuRC using immunogold labeling (Keating and Borisy, 2000). To define the ultra-structural localization of Xgrip210, we used the same method and identical conditions to localize Xgrip210 (Keating and

Borisy, 2000). MT were nucleated in CSF-arrested *Xenopus* egg extracts and immunostained using antibodies against Xgrip210 conjugated directly to 10-nm gold particles (Keating and Borisy, 2000). Of the γ TuRC-capped MT ($n = 136$ from three experiments), $42 \pm 7\%$ had one or more gold particles at the capped end.

To obtain finer resolution, we selected only those capped MT that had one gold particle at the end (Fig. 4 A). This procedure avoided including gold particles that were present as part of a cluster and not directly contacting Xgrip210. For each MT, the position of the center of the gold particle was measured relative to the end of the MT lattice. The positions of the gold particles were plotted and are shown superimposed on the image of a typical capped MT end (Fig. 4 B). Examination of individual MT ends revealed that a large proportion of gold particles were found at or near the pointed end of the capped MT (Fig. 4 B), a position corresponding to the cap structure of the γ TuRC. More than two thirds (71%) of the gold particles were within 15 nm of the

A

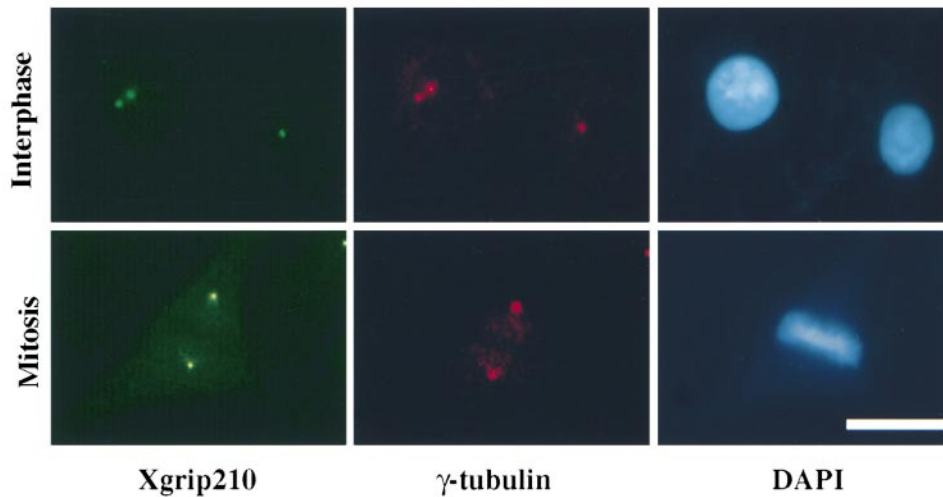
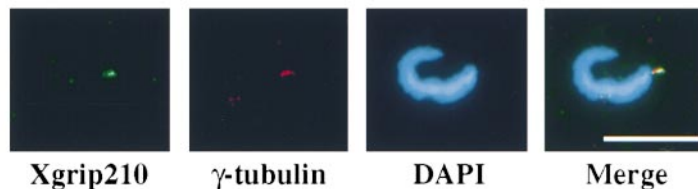


Figure 3. Xgrip210 colocalizes with γ -tubulin at the centrosome. (A) Immunofluorescence staining of *Xenopus* tissue culture cells with antibodies against *Xenopus* γ -tubulin and Xgrip210. (B) Immunofluorescence staining of *Xenopus* sperm centrosomes with antibodies against *Xenopus* γ -tubulin and Xgrip210. Nuclear DNA in A and B was stained by DAPI. Scale bars: 20 μ m.

B



tip of the γ TuRC cap, a distance at which the projecting antibodies could still contact Xgrip210 at the tip of the cap, suggesting that Xgrip210 is in this region.

We then compared the gold particle distribution of Xgrip210 to that of γ -tubulin, determined previously (Keating and Borisy, 2000; Fig. 4 B). The anti-Xgrip210 gold particles were further from the end of the MT lattice and closer to the central axis of the MT than those directed against γ -tubulin (Fig. 4 B and C). The average distance of the Xgrip210 gold particles from the end of the MT lattice was 10.4 ± 8.8 nm ($n = 69$), which is significantly different ($P < 0.05$, independent t test) from the average distance for the γ -tubulin gold particles (3.7 ± 9.9 nm, $n = 39$; Keating and Borisy, 2000). This can also be seen by comparing the histograms of the distances of the gold particles from the base of the MT lattice (Fig. 4 C), which shows that the distribution of anti-Xgrip210 particles is shifted further away from the end of the MT lattice than the anti- γ -tubulin particles. We have also measured the distance between the base of the microtubule lattice and the tip of the γ TuRC cap from 33 microtubules and found it to be 14.3 ± 3.7 nm. The distance distribution of the tip of the cap overlapped with the majority of the Xgrip210 gold particles (Fig. 4, C and D). Structural studies of γ TuRC suggested that MTs nucleated by γ TuRC grow from the face of the γ TuRC that is not covered by the cap structure (Keating and Borisy, 2000; Moritz et al., 2000). Therefore, the localization of Xgrip210 is consistent with the idea that Xgrip210 is a component of the γ TuRC cap structure that is distal to the base of MTs.

Xgrip210 Is Essential for MT Nucleation from the Sperm Centrosome

To study whether Xgrip210 is essential for γ TuRC function, we used assays described previously (Martin et al., 1998) to examine whether *Xenopus* γ TuRC can support centrosome assembly and MT nucleation in the absence of Xgrip210. *Xenopus* egg extracts support the assembly of a functional sperm centrosome in the presence of an ATP regenerating system in vitro (Felix et al., 1994; Stearns and Kirschner, 1994). We previously showed that immunodepleting γ TuRC from the egg extract completely inhibits sperm centrosome assembly. This can be reversed by adding purified *Xenopus* γ TuRC or partially purified *Xenopus* γ TuRC obtained in the pellet after precipitation of egg extract proteins with 30% ammonium sulfate (Martin et al., 1998). γ TuRC precipitated with 30% ammonium sulfate could be dissociated with 1 M salt and reassembled upon removal of the salt, producing functional γ TuRC capable of complementing the γ TuRC-depleted egg extract to support sperm centrosome assembly (Martin et al., 1998).

If Xgrip210 is essential for γ TuRC function, its removal from the γ TuRC should abolish the ability of the remaining γ TuRC components to complement the γ TuRC-depleted egg extract in the centrosome assembly assays. To test this, we precipitated *Xenopus* γ TuRC from the egg extract with 30% ammonium sulfate, resuspended the pellet in buffer containing 1 M salt, and immunodepleted Xgrip210 with anti-Xgrip210 antibodies. Control reactions were immunoprecipitated with random rabbit IgG. Fol-

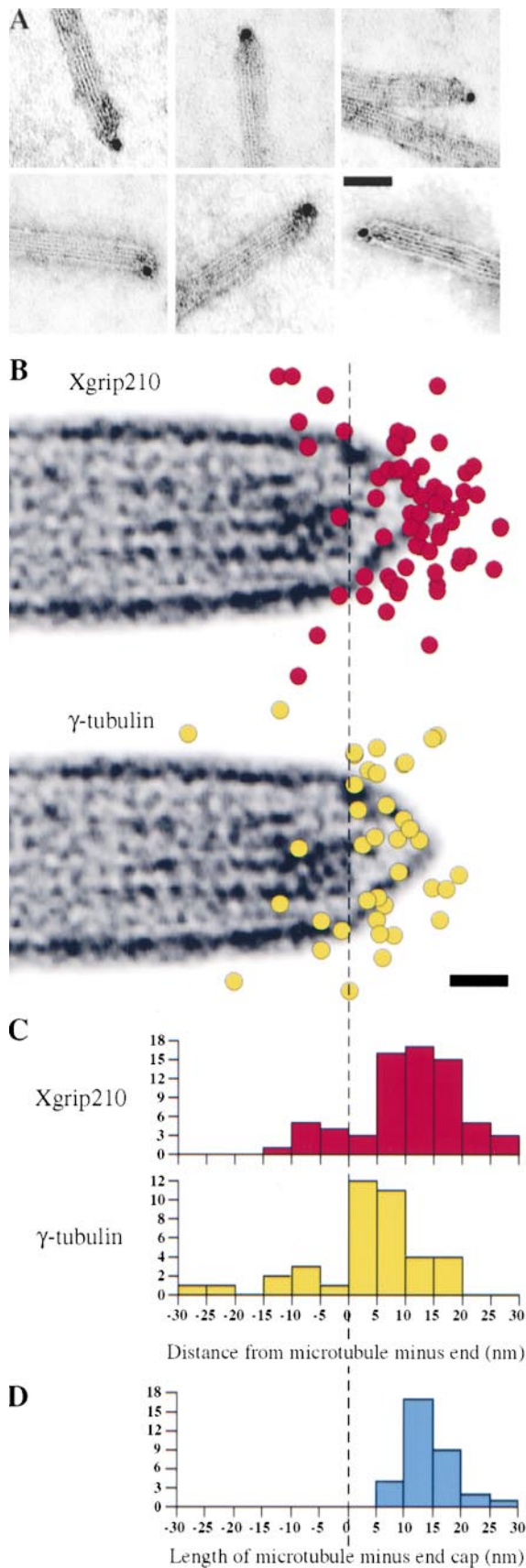


Figure 4. Immunogold localization of Xgrip210. (A) Examples of MTs with single 10-nm anti-Xgrip210 gold particles at the MT ends capped by γ TuRC. In all cases shown, the center of the gold particle was within 15 nm of the tip of the end cap. Scale bar: 50

lowing salt removal and concentration, the protein mixture was used to complement the γ TuRC-depleted *Xenopus* egg extract in centrosome assembly assays. Western blot analysis of the supernatant and pellet of the immunoprecipitations showed that most of the Xgrip210 (>70%) was removed by anti-Xgrip210 antibodies. There was no significant reduction of γ -tubulin or Xgrip109 compared with random IgG control, because Xgrip210 antibody did not coimmunoprecipitate Xgrip109 or γ -tubulin in high salt (Fig. 5, A and B). Centrosome assembly assays showed that removal of Xgrip210 almost completely blocked the formation of sperm asters (Fig. 5, C and D). This suggests that Xgrip210 was required for γ TuRC function.

Xgrip210 Is Essential for the Recruitment of γ -Tubulin and Xgrip109 to the Centrosome

To determine whether γ -tubulin and Xgrip109 were recruited to the centrosome in the absence of Xgrip210, we carried out the immunoprecipitation and centrosome assembly assays as described above except that nocodazole was added to the reactions to prevent MT formation. We found that it was easier to visualize the immunofluorescence staining of sperm centrosomes in the absence of MT asters. Neither γ -tubulin nor Xgrip109 localized to the sperm tips (where the sperm centrosomes are located) in the absence of Xgrip210 (Fig. 6).

Xgrip210 Is Not Localized at the Sperm Centrosome in the Absence of Xgrip109

Next, we asked whether Xgrip210 could localize to the centrosome in the absence of Xgrip109. The experiment was performed as described above, except that Xgrip109 was depleted. We found that there was no significant coprecipitation of Xgrip210 with Xgrip109 (Fig. 7, A and B). However, consistent with our previous finding (Martin et al., 1998), ~50% of total γ -tubulin coprecipitated with Xgrip109 (Fig. 7, A and B). Immunofluorescence staining with anti-Xgrip210 antibodies revealed that Xgrip210 failed to localize to the sperm tip in the absence of Xgrip109 (Fig. 7, C and D). Therefore, Xgrip109 is necessary for the localization of Xgrip210 to the sperm centrosome.

Xgrip210 Is Essential for the Assembly of the γ TuRC

We then examined whether Xgrip210 is essential for γ TuRC assembly. γ TuRC was partially purified by 30% ammonium sulfate and dissociated with high salt. Xgrip210 was immunoprecipitated from the salt-dissociated γ TuRC

nm. (B) Distribution of anti-Xgrip210 or anti- γ -tubulin gold particles superimposed on an image of a typical γ TuRC-capped MT end. Scale bar: 10 nm. (C) Distance distribution of the gold particles to the base of the MT lattice (dashed line), measured along the long axis of the MTs (red, Xgrip210; yellow, γ -tubulin). The average distance of the anti-Xgrip210 and anti- γ -tubulin gold particles from the base of the MT end are 10.4 ± 8.8 nm ($n = 69$) for Xgrip210 and 3.7 ± 9.9 nm ($n = 39$; Keating and Borisy, 2000) for γ -tubulin. (D) Distribution of lengths of the microtubule end cap measured from the end of the microtubule lattice (B, dashed line) to the tip, from a representative sample of microtubules. The average length of the cap was 14.3 ± 3.7 nm ($n = 33$).

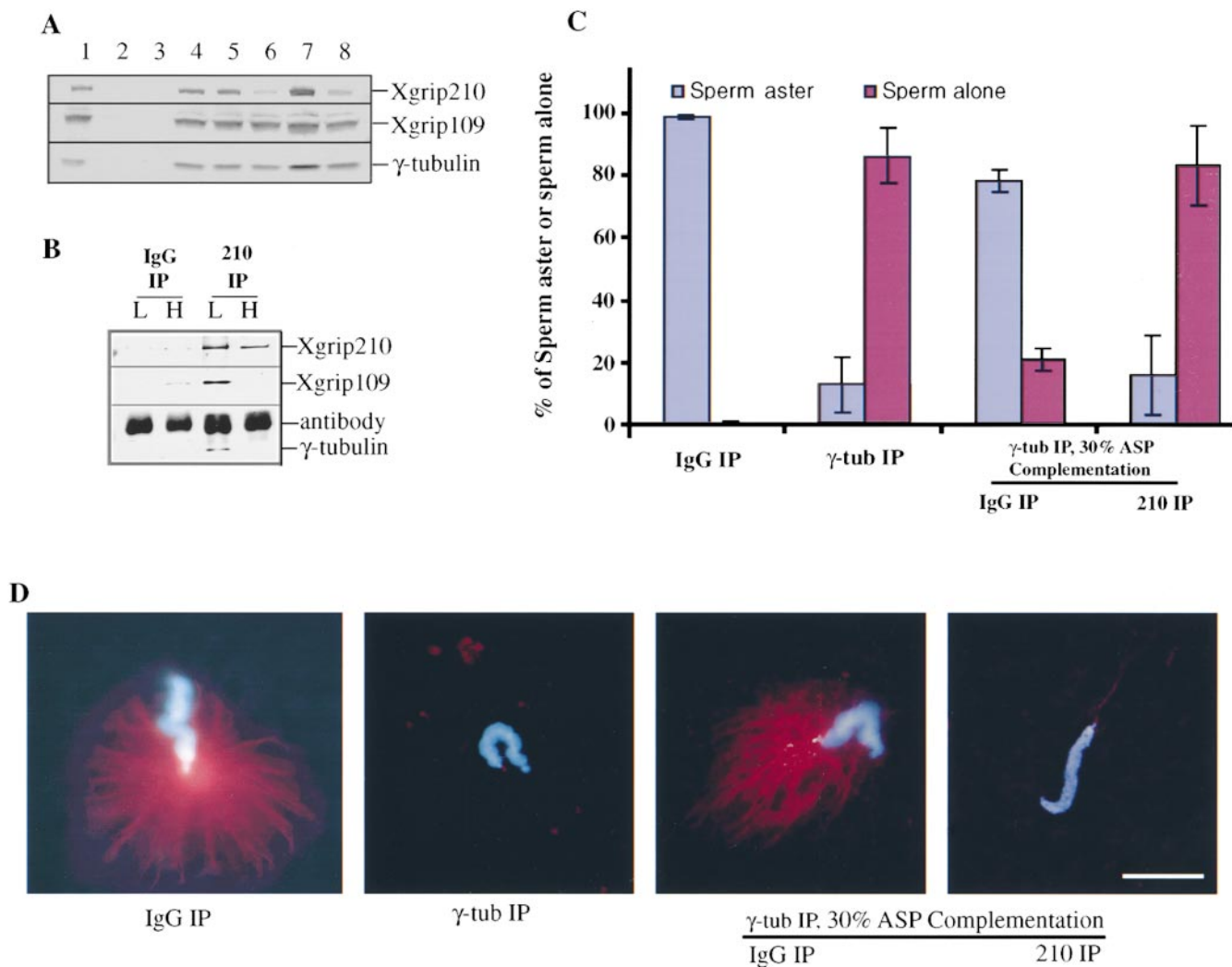


Figure 5. Immunodepletion of Xgrip210 inhibits sperm centrosome formation. (A) Western blot analysis of immunodepleted egg extracts and 30% ammonium sulfate fractions. *Xenopus* egg extract immunodepleted with either random IgG (1) or antibodies against *Xenopus* γ -tubulin (2). 30% ammonium sulfate supernatant (3) and pellet (4). 30% ammonium sulfate pellet (30% ASP) resuspended in HEPES 1M and immunodepleted with either random IgG (5) or antibodies against Xgrip210 (6). 7 and 8 are the same as 5 and 6, respectively, except that the proteins were desalted into HEPES 100 and concentrated \sim 10-fold. (B) Immunoprecipitates of random IgG (IgG IP) and Xgrip210 antibody (210 IP) from high (H) or low (L) salt-treated 30% ASP were analyzed by Western blot, probing with antibodies against Xgrips210 or 109, or γ -tubulin. Xgrip210 antibody did not immunoprecipitate significant amounts of Xgrip109 or γ -tubulin in high salt conditions, respectively. Immunodepletion of the egg extract with random IgG did not inhibit the formation of sperm centrosomes (IgG IP). The inhibition of sperm aster formation by immunodepleting γ -tubulin (γ -tub IP) was relieved by complementing with the 30% ASP that was depleted with random IgG (IgG IP), but not by the 30% ASP that was depleted with antibodies against Xgrip210 (210 IP). Error bars represent standard deviations calculated from three independent experiments. (D) Representative sperm nuclei with or without a MT aster from the assays in C are shown. Scale bar: 20 μ m. The MTs were labeled by inclusion of a small amount of rhodamine-tubulin in the assays, and the sperm DNA was stained with DAPI.

with random IgG precipitation as controls. After immunoprecipitation, the protein solutions were desalted and analyzed by sucrose gradient sedimentation followed by Western blotting to detect different γ TuRC components. Treatment with 1 M salt completely disassembled the γ TuRC (compare Fig. 8, A with E). Immunodepletion of Xgrip210 blocked γ TuRC reassembly from the salt-dissociated components (Fig. 8 D). In contrast, random IgG treatment allowed the reassembly of a fraction of the γ TuRC (Fig. 8 C). These results suggest that Xgrip210 is required for the formation of the γ TuRC. A fraction of Xgrip210 was aggregated by the high salt treatment (Fig. 8 A). How-

ever, under the same conditions, neither γ -tubulin nor Xgrip109 aggregated; instead, they cosedimented with an S value expected for that of the γ TuSC (Fig. 8, A and B). This suggests that Xgrip210 was not required for the formation of γ TuSC (Fig. 8, B and D, see also Discussion).

Discussion

Since the discovery of γ TuRC, a general picture has emerged regarding the organization of this complex. The tetrameric subcomplex γ TuSC appears to be the major building block of the lock washer-shaped ring that is cov-

A

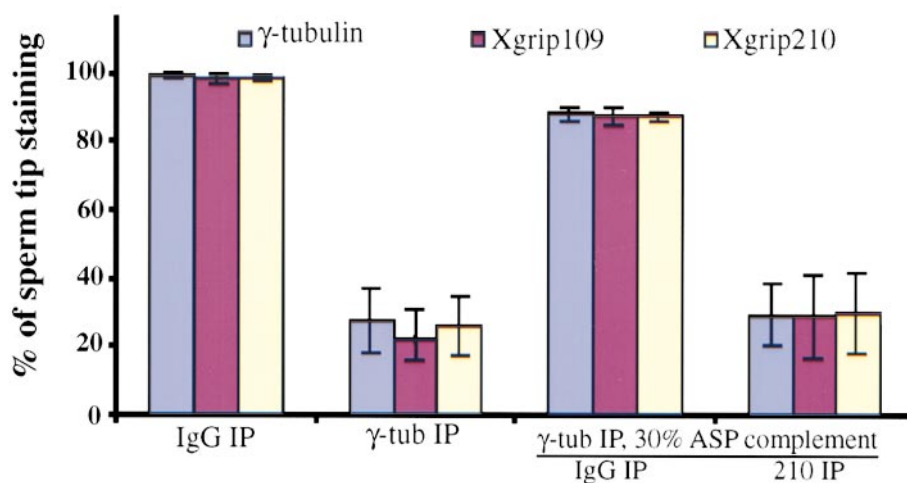
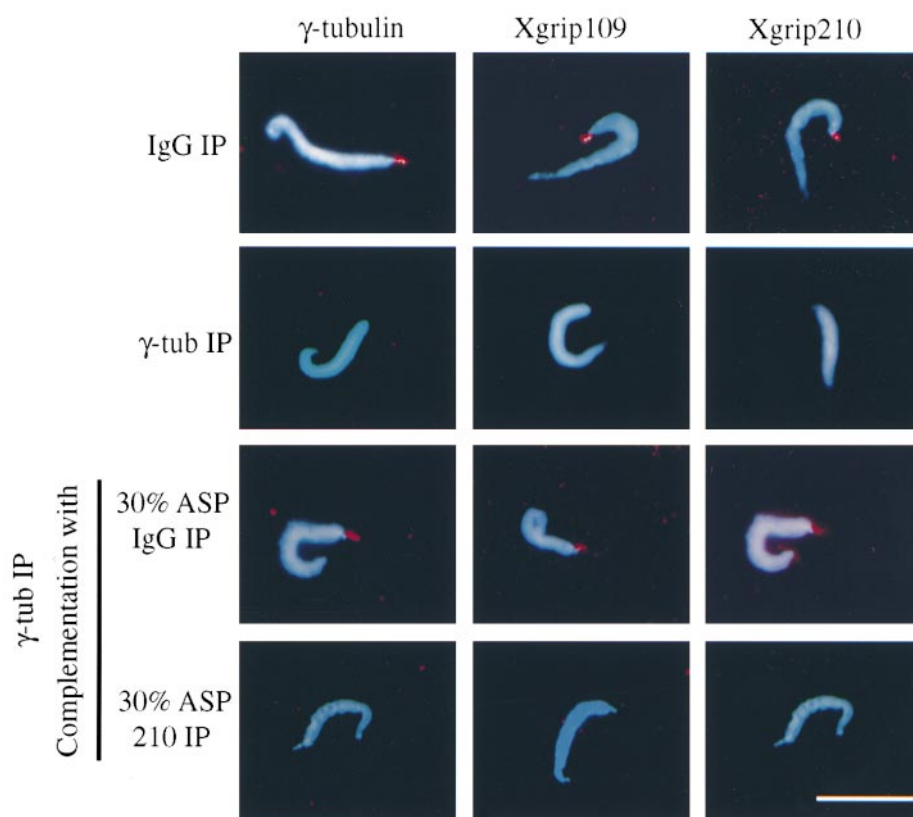


Figure 6. Immunodepleting Xgrip210 inhibits the recruitment of Xgrip109 and γ -tubulin to the sperm centrioles. (A) Blue, red, and yellow columns represent the percentage of sperm tips having γ -tubulin, Xgrip109, or Xgrip210 staining, respectively. Immunodepletion of γ -tubulin from *Xenopus* egg extracts (γ -tub IP) resulted in $\sim 70\%$ reduction of the recruitment of γ -tubulin, Xgrip109, or Xgrip210 to the sperm tips, while the same treatment with random IgG (IgG IP) had no effect. Complementing the γ -tubulin-depleted egg extract with the 30% ASP immunodepleted with random IgG (IgG IP) restored the localization of γ -tubulin, Xgrip109, and Xgrip210 to the sperm tips, while the same treatment with anti-Xgrip210 antibodies (210 IP) had no effect. Error bars: standard deviations calculated from three independent experiments. (B) Merged images of sperm incubated in extract immunodepleted with either random IgG (IgG IP) or anti- γ -tubulin antibodies (γ -tub IP), and γ -tubulin-depleted extract complemented with either mock-depleted (30% ASP IgG IP) or Xgrip210-depleted (30% ASP 210 IP) 30% ASP. The images were pseudocolored. Sperm DNA was stained with DAPI and merged with the antibody staining of the sperm tip. In cases where the images appear to be similar, it is due to double labeling of γ -tubulin (mouse monoclonal antibody; Sigma-Aldrich) and Xgrip210 (rabbit polyclonal antibodies). Scale bar: 20 μ m.

B



ered by a distinct cap on one face of the ring (Oegema et al., 1999; Keating and Borisy, 2000; Moritz et al., 2000). The cap structure was hypothesized to consist of the non- γ TuSC grips (Moritz et al., 2000). In *Xenopus* γ TuRC, these grips are Xgrips210, 133, and 75s. Clearly, characterizing the function of these grips is essential to understanding γ TuRC assembly and function. However, little progress has been made because the protein sequences of most of these grips were unknown. We have now cloned one of these subunits, Xgrip210. Probing the functions of Xgrip210 biochemically and structurally has provided important insights into the mechanism of γ TuRC assembly and recruitment.

Xgrip210 and γ TuRC Assembly

When *Xenopus* γ TuRC was dissociated with 1 M salt, Xgrip109 and γ -tubulin comigrated on sucrose gradients with an S value of ~ 11 S, similar to that of *Drosophila* γ TuSC (Fig. 8). Since approximately six molecules of γ TuSC are assembled into one γ TuRC, the multiple γ TuSCs might be held together by the non- γ TuSC subunits, Xgrips210, 133, and 75s to form the γ TuRC. Indeed, we found that Xgrip210 is required for γ TuRC assembly (Fig. 8). However, based on our studies of its homologue, Dgrip163, we believe that Xgrip210 alone would not be

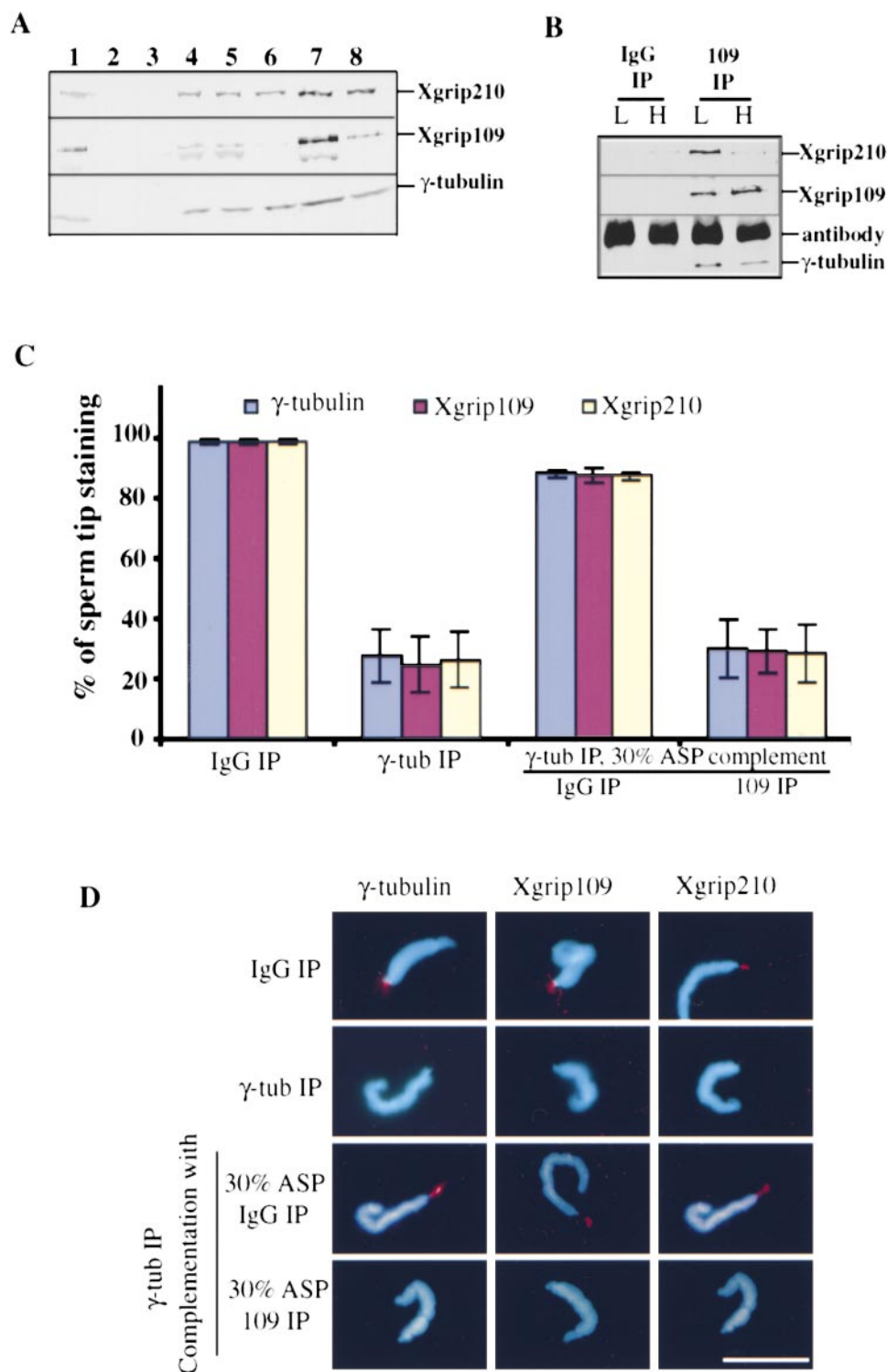


Figure 7. Immunodepletion of Xgrip109 inhibits the recruitment of Xgrip210 to the sperm centrioles. (A) Western blot analysis of the extracts and fractions used in the assays. *Xenopus* egg extract immunodepleted with either random IgG (1) or antibodies against *Xenopus* γ -tubulin (2). 30% ammonium sulfate supernatant (3) and pellet (4). 30% ASP resuspended in Hepes 1M and immunodepleted with either random IgG (5) or antibodies against Xgrip109 (6). 7 and 8 are the same as 5 and 6, respectively, except that the proteins were desalted into Hepes 100 and concentrated \sim 10-fold. (B) Immunoprecipitates of random IgG (IgG IP) and Xgrip109 antibody (109 IP) from high (H) or low (L) salt-treated 30% ASP were analyzed by Western blot, probing with antibodies against Xgrips210, 109, or γ -tubulin. Xgrip109 antibody did not coimmunoprecipitate a significant amount of Xgrip210 in the high salt conditions. (C) Blue, red, and yellow columns represent the percentage of sperm that have γ -tubulin, Xgrip109, and Xgrip210 staining at the tips, respectively. Immunodepletion of γ -tubulin from *Xenopus* egg extracts (γ -tub IP) resulted in $>$ 70% reduction of the recruitment of γ -tubulin, Xgrip109, or Xgrip210 to the sperm tips, while the same treatment with random IgG (IgG IP) had no effect. Complementing the γ -tubulin-depleted egg extract with the 30% ASP immunodepleted with random IgG (IgG IP) restored the localization of γ -tubulin, Xgrip109, and Xgrip210 to the sperm tips, while the same treatment with anti-Xgrip109 antibodies (109 IP) had no effect. Error bars: standard deviations calculated from three independent experiments. (D) Representative sperm from different treatments stained with antibodies against γ -tubulin, Xgrip109, or Xgrip210 are shown. In cases where the images appear to be similar, it is due to double labeling of γ -tubulin and Xgrip210. Scale bar: 20 μ m.

sufficient for the assembly of multiple γ TuSCs into γ TuRC (Gunawardane et al., 2000). Therefore, we propose that Xgrips210, 133, and 75s are all required for the formation of the cap structure and that this structure is important for γ TuRC assembly. Consistent with this idea, our ultrastructural studies revealed that Xgrip210 was most likely localized to the cap structure of the γ TuRC (Fig. 4).

Xgrip210 and the Recruitment of γ TuRC to the Centrosome

Several studies have strongly suggested that γ TuRC is recruited to the centrosome to nucleate MTs. However, how γ TuRC is recruited and docked at the centrosome remains unknown. We showed that Xgrip109 and γ -tubulin cosedimented with an S value expected for a γ TuSC on sucrose gra-

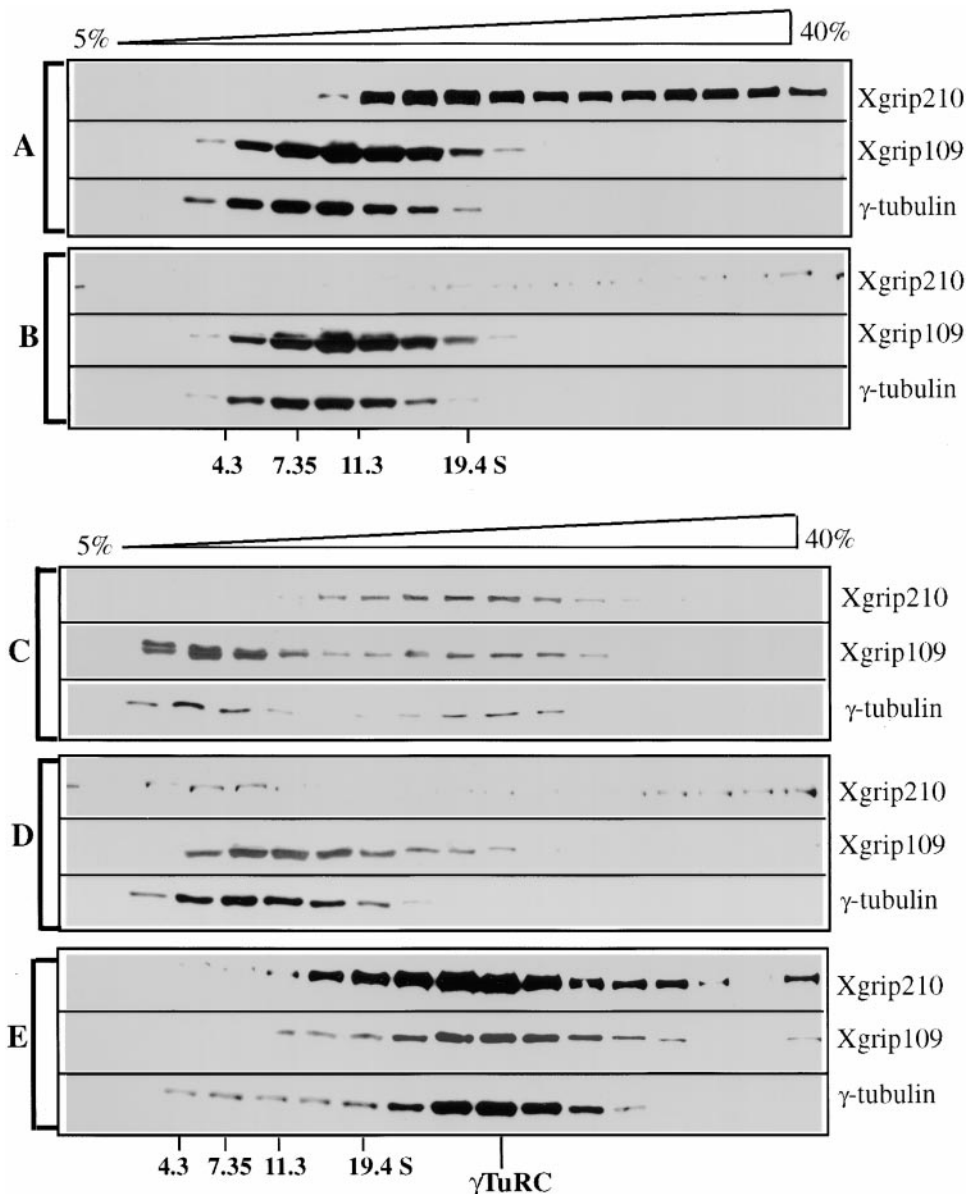


Figure 8. Xgrip210 is required for γ TuRC assembly. The γ TuRC present in 30% ASP was dissociated by Hepes 1M and immunodepleted with either random IgG (A) or Xgrip210 antibody (B) followed by analysis on 5–40% continuous sucrose gradients made in Hepes 100. Alternatively, after immunodepletion, salt concentrations were reduced by passing the immunodepleted mixtures through desalting columns equilibrated with Hepes 100, and then analyzed by sucrose gradient sedimentation (C and D). Random IgG depletion allowed the reassembly of a fraction of the Xgrip210, Xgrip109, and γ -tubulin into the γ TuRC-size complex (C), whereas immunodepleting Xgrip210 completely blocked reassembly (D). (E) 30% ASP was resuspended in Hepes 100 and fractionated on identical sucrose gradients as a control. All gradient fractions were analyzed by Western blot, probing with antibodies against Xgrips210 or 109, or γ -tubulin. The intact γ TuRC in E migrated to the same position as that of the reassembled γ TuRC shown in C. Protein standards used (S values indicated) were the same as described in Fig. 3 C.

dients in the absence of Xgrip210. However, neither Xgrip109 nor γ -tubulin was localized to the centrosome in the absence of Xgrip210, revealing, for the first time, that γ TuSC cannot bind to the centrosome on its own. Therefore, γ TuSC is unlikely to be responsible for tethering the γ TuRC to the centrosome. We also found that Xgrip210 cannot localize to the centrosome in the absence of Xgrip109. This suggests that Xgrip210 does not contain the localization signal or that the signal is insufficient for its localization.

Based on these findings, we propose the following two models to account for the recruitment and docking of the γ TuRC to the centrosome. In the first model, the recruiting/docking factor(s) could recognize the shape or a large surface area of the intact γ TuRC, but not the individual components. In this model, perturbations that disrupt the γ TuRC structure would block the recruiting/docking of all the γ TuRC subunits to the centrosome. Alternatively, one of the remaining uncharacterized γ TuRC subunits, Xgrip133 or Xgrip75s, could be involved in recruiting and docking the γ TuRC to the centrosome. In this case, Xgrip133

or Xgrip75s should be localized to the centrosome independent of the other γ TuRC subunits. Clearly, further studies of Xgrips133 and 75s will help us to differentiate these γ TuRC recruitment models.

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