In Vitro Reconstitution of Centrosome Assembly and Function: The Central Role of γ-Tubulin

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Summary

The centrosome nucleates microtubule polymerization, affecting microtubule number, polarity, and structure. We use an in vitro system based on extracts of Xenopus eggs to examine the role of γ-tubulin in centrosome assembly and function. γ-Tubulin is present in the cytoplasm of frog eggs and vertebrate somatic cells in a large ~25S complex. The egg extracts assemble centrosomes around sperm centrioles. Formation of a centrosome in the extract requires both the γ-tubulin complex and ATP and can take place in the absence of microtubules. γ-Tubulin is not present on the sperm prior to incubation in extract, but is recruited from the cytoplasm during centrosome assembly. The γ-tubulin complex also binds to microtubules, likely the minus end, independently of the centrosome. These results suggest that γ-tubulin is an essential component of the link between the centrosome and the microtubule, probably playing a direct role in microtubule nucleation.

Introduction

Microtubules are kinetically dynamic, noncovalent linear polymers of α- and β-tubulin. Although microtubules can assemble from purified subunits in vitro, microtubule assembly in cells is tightly controlled spatially and temporally. One level of control is that microtubules do not form freely in the cytoplasm under normal conditions. Instead, their assembly is nucleated by a microtubule-organizing center (reviewed by Brinkley, 1985). The centrosome is the major microtubule-organizing center in animal cells. It nucleates a discrete number of microtubules with a distinct polarity; the slowly growing (or minus) end of the microtubule is attached to the centrosome, and the rapidly growing (or plus) end is distal (Heidemann and McIntosh, 1980). The nucleated microtubules associated with the centrosome create a polarized, radial array of microtubules centered at the centrosome in interphase and contribute to the formation of a bipolar spindle in mitosis. Morphologically, there are two fundamental components of the centrosome: a pair of centrioles, themselves cylinders of nine triplet microtubules, and pericentriolar material, which is amorphous material surrounding the centrioles.

Microtubules nucleated by the centrosome appear to grow directly from the pericentriolar material (Gould and Borisy, 1977). The mechanism of nucleation is unknown, but it clearly does not occur by growth from preexisting microtubule ends. Many years of investigation into the nature of the pericentriolar material have not revealed anything morphologically resembling a microtubule template. Although a number of centrosomal proteins have been isolated (reviewed by Kalt and Schliwa, 1993), most have not been informative in understanding the molecular mechanism by which the centrosome nucleates microtubules. However, the discovery of γ-tubulin, a unique tubulin related to, but distinct from, α- and β-tubulin, in Aspergillus nidulans (Oakley and Oakley, 1989; Weil et al., 1986) and the subsequent demonstration that it is a conserved component of the centrosome or its equivalent in fungi and vertebrate cells (Oakley et al., 1990; Stearns et al., 1991; Zheng et al., 1991; Horio et al., 1991; Joshi et al., 1992) suggested a simple model for this aspect of centrosome function. In this model, γ-tubulin, strictly localized to the centrosome, would serve as the nucleator of microtubule growth, possibly by interacting directly with the αβ-tubulin heterodimer in a manner similar to the interaction between heterodimers during microtubule elongation. This model predicts that γ-tubulin would have an affinity both for the centrosome, presumably by virtue of an interaction with another centrosomal protein, and for the end of the microtubule that is at the centrosome, the minus end.

We have previously described the sequence of Xenopus laevis γ-tubulin and the localization of the protein to the centrosome of cultured cells from a number of species (Stearns et al., 1991). Here we describe the use of cell-free extracts made from Xenopus eggs to study the role of γ-tubulin in the assembly and function of the centrosome. Frog eggs provide several unique advantages for study of the centrosome. The unfertilized egg has no centrosomes but has all of the components required to assemble >1000 centrosomes without additional protein synthesis (Garrod et al., 1990). Assembly of the stored material into functional centrosomes can be initiated by the addition of templates to the egg, such as the sperm centrioles, as occurs during fertilization, or by injection of exogenous basal bodies (Heidemann and Kirschner, 1975). Concentrated cytoplasmic extracts from frog eggs are competent to carry out many of the reactions of which the intact cell is capable (Lohe and Masui, 1983; Lohe and Maier, 1985; Make-Lye and Kirschner, 1985; Murray and Kirschner, 1989; Sawin and Mitchison, 1991; Stearns and Kirschner, 1990). Using this system, we show that γ-tubulin is present in a large complex in the cytoplasm of eggs as well as somatic cells, that recruitment of this soluble γ-tubulin is essential for the formation of a centrosome, and that γ-tubulin is able to bind independently to both the centrosome and to the ends of microtubules. These results confirm predictions of the simple model and lead...
to the conclusion that γ-tubulin is a component of the microtubule-nucleating agent of the centrosome.

Results

Soluble γ-Tubulin Is Part of a Large Complex in Eggs and Somatic Cells

Xenopus eggs lack a centrosome until fertilization, when the sperm centrioles are converted into a centrosome, yet they contain all of the proteins necessary to construct many centrosomes (Gard et al., 1990). A quantitative Western blot of extract protein showed that γ-tubulin is about 0.01% of total protein, approximately the same level as in cultured animal cells. α- and β-tubulin are about 200-fold more abundant (Gard and Kirschner, 1987; Hiller and Weber, 1978; Stearns et al., 1991). To determine the nature of the soluble γ-tubulin, we sedimented proteins from egg extract on sucrose gradients. α- and β-tubulin sedimented as expected for the 6S heterodimer, whereas γ-tubulin sedimented as an ~25S complex (data not shown). There was no secondary peak of α- or β-tubulin at the γ-tubulin peak, indicating that the proteins are probably not associated in cytoplasm. Because the Xenopus egg is a specialized cell in which many types of proteins are stored for later use, we wished to determine the nature of γ-tubulin in somatic cells as well. Several cultured animal cell lines were examined, and in each case approximately half of the γ-tubulin was insoluble, presumably associated with the centrosome (Stearns et al., 1991); the remainder was soluble. Cytoplasmic extracts were made from XTC (Xenopus) and 293 (human) cells and sedimented on sucrose gradients. In both cases the γ-tubulin sedimented as a large complex, with approximately half of the γ-tubulin stably associated with this complex, sucrose gradient fractions were probed with antibodies against complex polypeptide 1 (TCP1), a component of the chaperone complex, as well as anti-γ-tubulin. The two proteins did not comigrate, indicating that they are probably not stably associated (data not shown). We have not determined whether γ-tubulin associates with the TCP1 complex or another chaperone complex during its synthesis.

In Vitro Assay for Centrosome Assembly and Function

Frog sperm have two centrioles, located in the sperm midpiece (Wilson, 1928), one of which serves as the basal body for the sperm flagellum. In vivo, the sperm centrioles initiate the formation of the first centrosome in the fertilized egg; this centrosome forms the large sperm aster soon after fertilization. To study centrosome formation and function in vitro, we devised an assay, based on that described by Sawin and Mitchison (1991a), in which demembranated Xenopus sperm, along with rhodamine-labeled bovine brain tubulin to visualize microtubules, were added to concentrated cytoplasmic extract of unfertilized X. laevis eggs. These extracts are arrested at the second division of meiosis, a state called cytosstatic factor (CSF) arrest; because of the similarity to mitosis, these are often referred to as mitotic extracts. The labeled tubulin was added to a level 10% that of the endogenous Xenopus tubulin. When these components were mixed together, sperm decondensation occurred within the first minute of incubation, and microtubule asters were visible after about 5 min incubation at room temperature (Figure 2), increasing in size with time. The observed asters were always at one end of the sperm, as would be expected for formation of the centrosome around the centrioles of the sperm midpiece. The capacity of the extract to generate asters on the sperm was similar to that reported for the egg in vivo, about 2000 per microliter of extract (Gard et al., 1990), and the per-
percentage of sperm bearing asters in non-saturating conditions was greater than 90%.

The cell cycle state of the extract affected the size and shape of the aster, but did not change the time course of aster formation in the extract. In CSF extract, which reflects the metaphase state, large, dense asters formed from the sperm centrosomes, and there was little free microtubule polymerization. In interphase extracts, the asters had fewer, longer microtubules, and there was substantial free microtubule polymerization. This is consistent with previous observations on the cell cycle changes in microtubule dynamics (Belmont et al., 1990; Verde et al., 1990). In CSF extracts, the microtubule aster changed in form during prolonged incubation, starting as an aster tightly associated with one end of the sperm, ultimately forming spindle-like structures, as described by Sawin and Mitchison (1991a).

Two properties of the centrosome formation reaction bear mentioning. First, there is an absolute requirement for extract; sperm nuclei that have not been exposed to extract are unable to nucleate an aster of microtubules in vitro (see below). Second, there is a time lag before any microtubule growth can be detected. This is not due to a lag in microtubule polymerization itself, as contaminating cell nuclei in the sperm preparation often have a centrosome associated with them and these centrosomes nucleate microtubules without any lag period (Figures 2C and 2D). To control for the possibility that sperm merely need to be expanded from their compact state before they can nucleate microtubules, we incubated the sperm nuclei in boiled egg extract. The activity that results in sperm expansion is stable to boiling (Newport and Dunphy, 1992). Sperm incubated in the boiled extract expanded within 1 min but were unable to nucleate microtubules in the assay described below and still showed a lag phase before aster formation when incubated in fresh extract.

**Separation of Recruitment from Nucleation**

The requirement for extract and the time lag associated with the formation of an aster from the sperm nuclei suggested that there are two steps to the process. First, centrosomal components are recruited from the extract...
Figure 3. Separation of Recruitment of Centrosomal Components and Nucleation of Microtubules

Sperm were first incubated in extract containing nocodazole, allowing the centrosome to form but preventing growth of microtubules from the centrosome. These sperm were then sedimented onto coverslips, washed, and incubated with pure brain tubulin containing a small amount of rhodamine-labeled tubulin to allow nucleation of an aster to occur. The samples were fixed and stained with DAPI to visualize the sperm chromatin. The recruitment reaction took place in CSF extract (A and B), buffer alone (C and D), extract with 5 mM EDTA added (E and F), or extract with 5 mM EDTA and 5 mM MgCl2 added (G and H). Scale bar, 5 μm.

Separation of recruitment from nucleation allowed an analysis of the requirements for the assembly of the centrosome free of the constraint of maintaining conditions for microtubule polymerization. We found that there is an ATP requirement for the formation of a centrosome in the extract. Extract that had been gel filtered to remove small molecules or treated with 5 mM EDTA to chelate Mg2+ was unable to form a functional centrosome on the sperm midpiece. This block could be relieved by addition of ATP to the gel-filtered extract or 5 mM Mg2+ to the EDTA-treated extract (Figures 3E–3H).

Soluble γ-Tubulin Is Recruited from Cytoplasm to the Nascent Centrosome

Because sperm nuclei incubated in nocodazole-treated extract become competent for nucleation, it must be that any components of the extract that are required for nucleation are recruited during this incubation. We examined sperm nuclei for the presence of γ-tubulin before (Figure 4) and after (Figure 5) incubation in the nocodazole-treated extract by immunofluorescence. γ-Tubulin was not detectable on the sperm midpiece before incubation in the extract (Figures 4A and 4B). After incubation, however, there was strong staining of the sperm midpiece, corresponding to the region that nucleates microtubules (Figures 5A and 5B). The staining appeared on a time course similar to that of the acquisition of nucleation capacity, becoming maximal at about 10 min (data not shown). The γ-tubulin...
staining changed over time from the original granular staining of the midpiece, seen after 7 min (Figures 5A and 5B), to two dots, seen after 30 min (Figures 5C and 5D). We believe that this represents conversion of the sperm-nucleating center from a simple accumulation of microtubule-nucleating material to two mature centrosomes, each with one of the original sperm centrioles. Depletion of ATP as described above completely blocked the recruitment of γ-tubulin.

Other centrosomal antigens, chosen on the basis of their localization to different parts of the centrosome, were examined in the manner described for γ-tubulin. The antigens fell into two classes, those present on the sperm midpiece even before incubation and those initially not present but recruited during the incubation, like γ-tubulin. Pericentrin, a large centrosomal protein localized to the pericentriolar material (Doxsey et al., 1994 [this issue of Cell]); centrin, a small calcium-binding protein related to T tubulin; and PM2, a protein involved in the assembly of microtubules, were also examined. The results are shown in Figures 5A–5D and 5F–5I. The left column shows DAPI fluorescence, and the right column shows immunofluorescence of the indicated antigen. Scale bar, 5 μm.
Figure 5. Centrosomal Antigens on Xenopus Sperm after Recruitment Reaction

Sperm were incubated in nocodazole-containing extract for 7 min (except for [C] and [D], which are 30 min) and then fixed and stained with antibodies against γ-tubulin (A-D), MPM-2 (E and F), pericentrin (G and H), centrin (I and J), and α-tubulin (K and L). The left column shows DAPI fluorescence and the right column immunofluorescence of the indicated antigen. Scale bar, 5 μm.
Figure 6. Localization of Two Classes of Centrosomal Antigens in Tetrahymena

Tetrahymena cells were fixed and stained with antibodies against α-tubulin (A), pericentrin (B), and γ-tubulin (C). Scale bar, 10 μm.
the yeast CDC37 gene product and found associated with the basal bodies in Chlamydomonas (Baron and Salisbury, 1988; Huang et al., 1988; we have cloned the gene for Xenopus centrin, and it is similar to the known algal centris [T. S., J. Salisbury, and M. K., unpublished data]); and α-tubulin, a component of the centriole microtubules were all present both before and after incubation, their appearance unchanged by extract incubation. Both α-tubulin and centrin appeared to be localized directly to the sperm centrioles, visible as two small dots in the sperm midpiece (Figures 4G–4J; Figures 5I–5L). In addition to the two small centrioles, α-tubulin staining sometimes showed a flagellar axoneme fragment adjacent to one of the centrioles (Figure 4J). Pericentrin stained a region of the midpiece that was similar to the γ-tubulin staining after incubation, but usually somewhat smaller in extent (Figures 4E and 4F; Figures 5C and 5H). In contrast, MPM-2, an antibody against a phosphoprotein epitope that increases dramatically in mitosis (Davis et al., 1983) but that is also found in microtubule-organizing centers in both mitosis and interphase, showed a staining pattern identical to that of γ-tubulin: no staining of the sperm nucleus prior to incubation in extract or after incubation in extract depleted of ATP, but strong staining after extract incubation, coincident with the acquisition of nucleating capacity (Figures 4C and 4D; Figures 5E and 5F). These results indicate that the sperm midpiece already has a number of the proteins that are present in mature centrosomes, possibly only requiring a few extract components, γ-tubulin and a phosphorylated epitope recognized by MPM-2 among them, to make a functional centrosome.

This distinction among centrosomal antigens is conserved, as determined by examining the localization of α-tubulin, γ-tubulin, and pericentrin in the ciliated protozoan Tetrahymena thermophila (Figure 6). Tetrahymena has a cortical microtubule cytoskeleton, basal bodies along its entire length, and complex microtubule structures associated with its oral apparatus. α-Tubulin was present in all of the microtubule structures (Figure 6A). γ-Tubulin was present only in a large structure at the base of the oral apparatus that corresponds to the previously described r-shaped region (Numata et al., 1983) and that has been observed to stain with other centrosomal antigens in other ciliates (Figure 6C). This is likely to be a cytoplasmic microtubule-nucleating site (Keryer et al., 1990). MPM-2 staining appeared identical to γ-tubulin staining (data not shown). Pericentrin was present on both the basal bodies and the oral apparatus (Figure 6D).

**γ-Tubulin Is Required for Centrosome Formation**

The above results showed that γ-tubulin is associated with the sperm centrosome and that appearance of γ-tubulin on the nascent centrosome correlates with the acquisition of nucleation capacity. To determine whether γ-tubulin is required for the formation of a functional centrosome, we used two independent methods to inhibit γ-tubulin function. The first made use of the large size of the γ-tubulin-containing complex. Egg extract was centrifuged at 400,000 × g for 1 hr to sediment most of the γ-tubulin complex (Figure 7B). Most of the α-tubulin remained in the supernatant under these conditions, and the extract remained competent to polymerize microtubules (data not shown). This centrifuged extract was completely deficient in assembly of a sperm centrosome when compared with an extract that had been centrifuged at 100,000 × g (Figure 7A). If the pellet was added back to the supernatant, centrosome-forming activity was restored. Second, we added polyclonal γ-tubulin antibody XG-2-5 to extract for 1 hr on ice and then assayed the ability of the extract to support sperm centrosome formation. Incubation with the antibody completely inhibited the formation of a sperm centrosome, whereas control incubations with nonimmune rabbit immunoglobulin G or XG-2-5 inactivated by heating had no effect. Together, these experiments provide evidence that γ-tubulin, in the form of the 2S complex, is required for the formation of a centrosome in vitro.

**The γ-Tubulin Complex Associates with the Minus End of Microtubules**

In extracts made from CSF-arrested eggs, free microtubules do not form. It has been shown, however, that if microtubule polymerization is forced by adding low concentrations of the microtubule-stabilizing drug taxol, the microtubules will organize into asters in the absence of any added centrosomes or centrioles (Verde et al., 1991). A aster formation is dependent on the mitotic state and requires the minus end–directed microtubule motor cytoplasmic dynein, suggesting that the microtubules in such asters are arranged with their minus ends at the center, as in centrosomal asters. We have noticed a similar phenomenon when dimethyl sulfoxide (DMSO), which also enhances microtubule polymerization, is added to egg extracts to a concentration of 5%. Approximately 20 min after addition of DMSO, distinct asters were present in the CSF extract, whereas only single microtubules or small bundles of microtubules were present in the interphase extract (data not shown). With increasing time, the asters in the CSF extract became larger, often losing their astral morphology and becoming more polar; microtubules in interphase extract remained disorganized. To determine whether γ-tubulin was localized to the ends of microtubules even in the absence of centrosomes, DMSO asters were fixed and treated for immunofluorescence with anti-γ-tubulin antibodies. There was staining at the center of each aster as well as punctate staining with increasing distance from the center (Figures 8A and 8B). This suggested that γ-tubulin is able to associate with the minus ends of microtubules made in solution. These asters were also examined for the presence of other centrosomal antigens. Both pericentrin (Figures 8C and 8D) and MPM-2 (data not shown; Verde et al., 1991) also localized to the center of the DMSO asters, whereas centrin, which appears to be closely associated with basal bodies and centrioles, rather than nucleating material per se, did not (Figures 8E and 8F). Exactly the same localization results were obtained when asters were made with taxol, as described by Verde et al. (1991).

To confirm the immunofluorescence results biochemically, DMSO asters were assembled and pelleted through a glycerol cushion, and the pelleted protein was examined...
Figure 7. Requirement for High Molecular Weight Fraction Containing γ-Tubulin for Centrosome Formation

(A) Egg extract was centrifuged at either 100,000 x g or 400,000 x g and assayed for the ability to form a centrosome from sperm centrioles as in Figure 2. Rhodamine tubulin fluorescence is on the left and DAPI staining of the sperm chromatin on the right.

(B) The presence of γ-tubulin in the 100,000 x g supernatant (S100), the 400,000 x g supernatant (S400), and the 400,000 x g pellet (P400).

by Western blotting for the presence of γ-tubulin and centrin. As a control, DMSO plus nocodazole was added to extract and treated as above. α- and β-tubulin, visible as the major Coomassie blue-stained bands, pelleted in the DMSO-treated extract, but not in the DMSO plus nocodazole-treated extract (Figure 9). A fraction of the total γ-tubulin also pelleted in the DMSO-treated extract, but not in the DMSO plus nocodazole-treated extract. The protein loaded in the pellet fractions was equal to 5-fold that in the supernatant fractions on a volume basis; thus, the actual amount of γ-tubulin pelleted in this experiment is small compared with the total γ-tubulin. In contrast with γ-tubulin, centrin remained in the supernatant under both conditions, providing evidence that true centrosomes, which normally contain centrin and centrioles, are not forming de novo in the DMSO-treated extract, as they can in certain other amphibian egg extracts (Palazzo et al., 1992). This is in accord with the finding that taxol asters lacked centrioles (Verde et al., 1991). Interestingly, the same amount of γ-tubulin pelleted with microtubules when microtubule polymerization was stimulated with DMSO in interphase extract (data not shown). Asters do not form in interphase extract; thus, there is no specific requirement for an organized array of microtubules for γ-tubulin binding to microtubules.

Only a fraction of the α-, β-, and γ-tubulin pelleted in the DMSO aster experiment, possibly because the conditions that are optimal for aster formation do not drive all of the α- and β-tubulin into microtubule polymer. To determine whether it was possible to deplete the extract of γ-tubulin quantitatively, 1 μM taxol was added to extract to polymerize most of the α- and β-tubulin into microtubules, which were then pelleted through a glycerol cushion. Almost all of the γ-tubulin pelleted with the microtubules when taxol was used to drive assembly (data not shown). Because most γ-tubulin in the extract is in the 95S complex, this complex must be able to associate with microtubules. We next tested whether γ-tubulin complex partially purified on a sucrose gradient could associate with microtubules. The peak γ-tubulin-containing fraction (~ 1 nM γ-tubulin) from a sucrose gradient similar to those shown in Figure 1 was mixed with taxol-stabilized microtubules of average length of 9.6 ± 4.5 μm, in a range of microtubule concentrations from 500 pM to 15 pM. Both GTP and taxol were present in the reaction mixtures in addition to buffer. Reactions were incubated at 30°C for 20 min and then pelleted.
through a glycerol cushion. At microtubule concentrations of 125 pM or above, greater than 90% of the γ-tubulin pelleted (supernatant data not shown), and ~50% pelleted with 60 pM microtubules (Figure 10).

Discussion

γ-Tubulin is a unique relative of α- and β-tubulin that does not assemble into microtubule polymers. Instead, γ-tubulin is mostly limited in distribution to the microtubule-organizing center, the centrosome in animal cells. We demonstrate in this paper that in eggs and somatic cells, γ-tubulin is also present in the cytoplasm as a high molecular weight complex of ~25S. This γ-tubulin particle is able to associate independently with a forming centrosome in the absence of microtubules and with microtubules in the absence of a centrosome. Recruitment of the γ-tubulin particle from the cytoplasm is an essential step in formation of a functional centrosome. These results suggest that γ-tubulin is a critical part of the link between the centrosome and the nucleated microtubule, likely playing a direct role in microtubule nucleation, and provide a possible explanation for the cell cycle regulation of the nucleation capacity of the centrosome.

It was not surprising that the frog egg has a substantial pool of soluble γ-tubulin; the rapid divisions of early development occur without new protein synthesis, and the egg must have the capacity to assemble >1000 centrosomes from stored components (Gard et al., 1990). It is interesting, then, that somatic animal cells, with no rapid division...
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requirement, also have a soluble pool of γ-tubulin, which includes about half of the total γ-tubulin. Though it seems unlikely that cells would require such a pool strictly for centrosomal synthesis, it is possible that the free γ-tubulin is important for modulation of the nucleation capacity of the centrosome. Mitotic centrosomes nucleate more microtubules than interphase centrosomes (Kuriyama and Borisy, 1981), and this change appears to be regulated by phosphorylation (Centonze and Borisy, 1990; Buendia et al., 1992). Increased nucleation might be accomplished by recruitment of the soluble γ-tubulin to the centrosome, possibly by a change in the phosphorylation state of a centrosomal component to which γ-tubulin binds, or of γ-tubulin itself. This model predicts that there would be less soluble γ-tubulin in mitotic cells than interphase cells, as more would be associated with the mitotic centrosome.

The soluble γ-tubulin is in a large ~25S complex in the cytoplasm of eggs as well as that of both frog and human cultured cells. We propose that this particle be termed the γ-some, in keeping with the naming of other large cytoplasmic particles. This complex is unlikely to be vesicular, as nonionic detergent had no effect on its sedimentation in sucrose gradients. The complex could be reversibly dissociated with salt, and the dissociated γ-tubulin was similar in size to the αβ heterodimer, although this was not precisely determined. It is unlikely that γ-tubulin is part of a heterodimer with either α- or β-tubulin in the γ-some, because all of the α- and β-tubulin in egg extract sedimented as expected for 6S heterodimer, with no second peak at the location of γ-tubulin in gradients. These results are particularly interesting in light of the work of Raff et al. (1993), who found that under conditions in which centrosomal proteins bind to a microtubule-affinity column, γ-tubulin in Drosophila extracts is part of a complex with two previously identified centrosomal proteins, DMAP190 and DMAP60 (Kellogg and Alberts, 1992; Kellogg et al., 1989). In their work, α- and β-tubulin did not bind to the column, again suggesting that γ-tubulin does not associate with α- and β-tubulin in the cytoplasm, and a portion of the γ-tubulin in crude extract comigrated in sucrose gradients with the DMAP60 protein. This complex was much smaller than the one described here, although the gradients were run in concentrations of salt that dissociate the particle that we have found.

The presence of soluble γ-tubulin in cytoplasm raises the questions of how such centrosomal components are assembled and how they are prevented from assembling inappropriately. A likely solution in animal cells is that the centriole provides the template for assembly of nucleating material, and without this template the γ-tubulin particle and other potential centrosome components never reach the critical mass required to form functional nucleating

protein  γ-tubulin centrin

a b c d MW a b c d a b c d

Figure 9. γ-Tubulin Sediments with Centrosome-Free Asters

DMSO asters were prepared with CSF extract as in Figure 8 and sedimented through a cushion, and the supernatant and pelleted proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue for total protein. Identical samples were treated for Western blotting and probed with EAD-2-4 anti-γ-tubulin antibody and with 20H5 anti-centrin antibody. As a control, DM60 plus 2 μM nocodazole was added to a separate aliquot of extract, which was then treated as for the DMSO alone. The lanes are supernatant of DMSO plus no nocodazole (a), supernatant of DMSO (b), pellet of DMSO plus nocodazole (c), and pellet of DMSO (d). The lane labeled MW is molecular weight markers, with sizes in kilodaltons indicated on the left of the figure. For the pellet samples, the amount of protein loaded represented 5-fold the amount of supernatant protein on a volume basis.

500 250 125 60 30 15

α, β-tubulin γ-tubulin

Figure 10. Partially Purified γ-Tubulin Particle Associates with Microtubules

Taxol-stabilized microtubules at the indicated concentrations (picomoles or micromoles) were mixed with the peak γ-tubulin-containing fraction from the sucrose gradient in Figure 1 and incubated for 20 min at 30°C. Microtubules were then pelleted. α- and β-tubulin are visualized by Coomassie staining (top panel), and γ-tubulin is visualized by probing a Western blot of pelleted protein with anti-γ-tubulin antibody (bottom panel). The concentration of γ-tubulin in the reaction was ~1 nM.
material. Although it could be argued that in our experiments the sperm midpiece might be specialized for the purpose of recruitment of nucleating material, it has been shown that isolated basal bodies or centrioles from a number of evolutionarily divergent sources will serve to initiate an aster in frog eggs (Heidemann and Kirchmer, 1975), presumably by recruiting nucleating material as demonstrated here for sperm centrioles. Clearly, nucleating material can be organized in other ways as well, as found in the experiments presented here and by Verde et al. (1991) with induced asters in mitotic egg extract, as well as in vivo experiments that have shown that animal cells are capable of regenerating a polar microtubule cytoskeleton after removal of the centrosome (Maniotis and Schliwa, 1991; McNiven and Porter, 1988).

By antibody staining of sperm centrosomes and Tetrahymena, we have demonstrated a theme common to both: basal bodies or centrioles have certain proteins associated with them, but it is only by the addition of y-tubulin (and possibly other proteins) that they become competent to nucleate microtubules other than those grown directly from their ends. The immunofluorescence experiments define three distinct classes of centrosomal proteins: those associated strictly with centrioles or basal bodies, such as a-tubulin and centrin; those associated with centrioles or basal bodies, but also with pericentriolar material, such as pericentrin; and those associated strictly with the pericentriolar-nucleating material, such as y-tubulin and an MPM-2 antigen. This suggests that there is some order to the assembly of centrosomal proteins, with the outermost proteins likely being directly involved in nucleation. Thus, one might imagine that a protein like pericentrin, with coiled-coil motifs typical of structural proteins (Doxsey et al., 1994), serves as a scaffold for the assembly of the y-tubulin that is required for microtubule nucleation.

In all of the cases that we have examined, y-tubulin staining appeared identical to that of MPM-2, an antibody against a phosphoprotein epitope, raising the possibility that y-tubulin itself is phosphorylated and becomes an MPM-2 antigen, possibly during the process of centrosome assembly. MPM-2 has been reported to stain mitotic centrosomes (Vandere et al., 1984), but we have found that it also stains interphase centrosomes, though less intensely (T. S. and M. K., unpublished data). Phosphorylation has been shown to be important in aspects of centrosome function in frog egg extracts (Buendia et al., 1991; Ohta et al., 1993). In all of our experimental treatments, the presence of y-tubulin and the MPM-2 antigen were correlated; all treatments that prevented the recruitment of one also prevented recruitment of the other. In experiments on the activation of Schizosaccharomyces pombe spindle pole bodies in Xenopus egg extract, Ma-suda et al. (1992) found that inactive spindle pole bodies stained with y-tubulin antibody but not MPM-2, whereas activated spindle pole bodies stained with both, suggesting that the presence of y-tubulin alone is not sufficient for microtubule nucleation. This could mean either that y-tubulin must be modified before it can be functional for nucleation or that another protein is required, although the heterologous nature of these experiments might not reflect in vivo requirements.

The y-tubulin complex is able to bind to microtubules in the absence of a centrosome, suggesting that the soluble complex might have a function in addition to serving as a reservoir for increasing centrosomal nucleation capacity. The stoichiometry of binding of the y-tubulin complex to microtubules and the localization of y-tubulin to the center of DMSO asters both suggest that the y-tubulin complex is binding the ends of microtubules, most likely the minus end. Because it is not known how many proteins are in the complex, it is not known whether y-tubulin is binding directly to the microtubule end or whether the binding is taking place through other components of the complex, but given that y-tubulin displays considerable homology to a- and b-tubulin, approximately the same as that found between a- and b-tubulin, it seems likely that y-tubulin is interacting directly. Although the composition of the y-some is not known, we can make high and low estimates for the concentration of particles in extracts and semipurified preparations, with the extremes being a single y-tubulin molecule per particle or a particle composed entirely of y-tubulin. From comparison to known quantities of bacterially expressed y-tubulin, we can estimate that the concentration of y-tubulin protein in the sucrose gradient fraction used to assess microtubule binding is ~1 nM. Thus, if y-tubulin binds to the minus ends of microtubules, the number of microtubule ends required to bind all of the y-tubulin in that experiment would be consistent with a particle containing ~10-20 y-tubulin molecules. y-Tubulin has a molecular weight of ~5 x 10^4 daltons, whereas the particle can be estimated to have a molecular weight of ~2 x 10^6 daltons, assuming that it is a globular complex. Thus, it is likely, based on these crude estimates, that there are other proteins in the complex.

Given the presence of both soluble and centrosomally bound forms of y-tubulin and that the y-some is able to bind to the ends of microtubules even when it is not in the context of a centrosome, we propose that in vivo every microtubule has a y-some at its minus end, even those in which the minus end is not at the centrosome. Consistent with this, we have found, by immunofluorescence, significant amounts of y-tubulin in the spindle (T. S. and M. K., unpublished data), appearing to spread from the poles, consistent with the known arrangement of microtubule ends in spindles (McDonald et al., 1992). It has also been observed that the minus ends of microtubules seem to be stable in vivo to the dynamics of polymerization and depolymerization that the plus end undergoes (Supranant, 1991; Tao et al., 1988), suggesting that there must be a minus end cap, which we would propose is the y-some. We imagine that when concentrated at the centrosome, the y-some serves to nucleate microtubule assembly by lowering the critical concentration for assembling the complex microtubule polymer lattice, possibly by acting as a template itself for the addition of a b heterodimers. Because the y-some is bound to the centrosome through other interactions, the resulting nucleated microtubule remains attached to the centrosome. Some of these microtubu-
bulbs may be released from the centrosome with the γ-tubulin minus end, allowing for a spatial reorganization of the microtubule cytoskeleton without altering the dynamics of polymerization and accounting for some of the microtubules that are not associated with the centrosome in many cells. It remains to be seen how this model of γ-tubulin binding to the minus ends of microtubules can be reconciled with the observed microtubule flux in the spindle (Mitchison, 1988; Sawin and Mitchison, 1991b), the existence of which indicates that the minus ends of microtubules in the mitotic spindle must be able to depolymerize. It is possible that the interaction of γ-tubulin with the microtubule minus end is relatively dynamic in the spindle. It will be particularly interesting to examine the purified γsome by electron microscopy to determine whether its structure reveals anything of the mechanism of binding to microtubules and to the centrosome.

**Experimental Procedures**

**Preparation of Egg and Cell Extracts**

X. laevis egg extracts were prepared from laid eggs as described (Murray and Kirschner, 1989) with the exception that the eggs were first packed by spinning at 2,000 rpm for 2 min in an SW28 rotor and then crushed by spinning at 20,000 rpm for 20 min. These are CSF extracts, naturally arrested at meiosis II. Interphase extracts were made from CSF extracts by adding CaCl₂ to 0.5 mM and cycloheximide to 100 μM and by incubating at room temperature for 45 min. Cultured cell extracts were made by adding extract buffer (XB: Murray and Kirschner, 1989) containing protease inhibitors and 0.5% Triton X-100 to a dish of cells that had been washed once with PBS, scraping cells from the dish, and centrifuging for 1 min in a microfuge at 4°C. X. laevis XIC cells were grown in 5% L-15 medium with 25 mM fetal calf serum at 25°C in ambient atmosphere. Human 293 cells were grown in DME H-21 with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere.

**Fluorescent Labeling of Tubulin**

Purified bovine brain tubulin was labeled with tetramethyl rhodamine by the high pH method as described (Hyman et al., 1991). The stoichiometry of labeling after two cycles of temperature-dependent assembly/disassembly was approximately two fluorochromes per tubulin dimer. The labeled tubulin was stored at 20 mg/ml in 50 mM potassium-gluconate, 0.5 mM MgCl₂ (pH 6.5) at 8°C.

**Preparation of Depleted Sperm Nuclear**

Sperm nuclei were isolated from X. laevis testes as described (Sawin and Mitchison, 1991a), except that the testes were macerated between two sintered glass surfaces. The sperm were stored in 250 mM sucrose, 15 mM HEPES (pH 7.4), 1 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.1% β-mercaptoethanol, 10 μg/ml each of leupeptin, pepstatin, and chymostatin with 0.3% BSA and 30% glycerol at 8°C. Aliquots of sperm could be thawed and refrozen at least three times without effect on their ability to support centrosome formation.

**Sucrose Gradient Fractionation**

Linear sucrose gradients (2.2 ml of 0%-40%) in XB were prepared in TLS-55 centrifuge tubes (Beckman, Incorporated), and 100 μl of egg extract diluted 1:1 with XB or 100 μl of soluble cell extract was layered on top. The gradients were spun at 55,000 rpm in a TLS-55 rotor for 2.5 hr at 4°C. Fractions (150 μl) were removed manually. Each fraction (40 μl) was loaded on an SDS-polyacrylamide gel. The position of α-, β-, and γ-tubulin in the gradients was determined by probing Western blots of the gels with DM1a, DM1b, and EAD-2 antibodies.

**Centrosome Assembly Reactions**

Reactions were usually carried out by mixing 10 μl of egg extract with 0.5 μl of demembranated sperm (~1000 sperm) and 0.5 μl of rhodamine-labeled bovine brain tubulin at a concentration of 2 mg/ml in a 200 μl tube and by incubating at room temperature (~24°C) for the indicated time. The presence of asters was assayed by removing a 1 μl aliquot and mixing with 1 μl of permeabilization buffer (PBS: 82 mM PIPES [pH 6.8], 5 mM EGTA, 1 mM MgCl₂, 0.5% Triton X-100) containing 1 μg/ml DAPI on a slide and covering with a 12 mm round coverslip. For documentation and for immunofluorescence of treated sperm, reactions were diluted 10-fold with PBS buffer and sedimented onto coverslips, as described (Swan et al., 1991b), through PBS with 20% glycerol and without Triton X-100 at 10,000 rpm in a Sorvall HS-6 rotor for 10 min. Nocodazole was added to the extracts to 2 μg/ml from a freshly prepared 20 μg/ml stock in XB. Some reactions were carried out with extract that had been subjected to a medium speed spin, 50,000 rpm in a Beckman TLA-100 rotor for 30 min. This did not affect the centrosome-forming capacity of the extracts and resulted in improved microscopy. High speed spins were 100,000 rpm in a TLA-100 rotor for 1 hr.

Recruitment and nucleation were separated by incubating sperm in extract containing 2 μg/ml nocodazole and then sedimenting the spHrili onto coverslips as described above. The sperm were then washed with BRB80 (80 mM PIPES [pH 6.8], 1 mM EGTA, 1 mM MgCl₂) and incubated with 2 mg/ml purified brain tubulin, 0.2 mg/ml rhodamine-labeled tubulin in BRB80 with 5 mM MgCl₂, 1 mM GTP, and 20% glycerol directly onto coverslip in a moist chamber at 37°C for 15 min. Microtubules were fixed by the addition of PB containing 5% glutaraldehyde for 10 min.

**Immunofluorescence**

Sperm were fixed for immunofluorescence by sedimenting onto coverslips as above and fixing for 5 min in cold methanol. Samples were then rehydrated with PBS and blocked with PBSBT (PBS with 3% BSA, 0.1% Triton X-100, and 0.2% azide) for 15 min. T. thermophila phosphotungstic acid was diluted in PBSBT and incubated with 1 μg/ml DAPI in PBS and coverslips were mounted in PBS (pH 9.0) with 90% glycerol and 1 mg/ml p-phenylenediamine. γ-Tubulin antibody XG-1-4 (Steams et al., 1991) was used for immunofluorescence. Anti-centrin monoclonal antibody 201 was the gift of J. Geilsbury, and anti-pericentrin antibody was the gift of S. Duxsey.

**Association of γ-Tubulin with Microtubules**

DMSO asters were made by adding DMSO to 5% to CSF extract containing rhodamine-tubulin. This was incubated for 30 min and then examined by fluorescence microscopy as described above. Taxol asters were made by adding taxol (a gift of V. L. Narayanan) to 0.1 μM CSF extract. For immunofluorescence microscopy, the asters were sedimented onto coverslips and processed as described above for sperm asters. Asters were prepared for Western analysis by diluting 10-fold with PBS buffer and pelleting through a cushion of BRB80 with 40% glycerol. The supernatant above the cushion and the pellet were both analyzed by SDS-PAGE. The ability of the γ-tubulin particle to bind to microtubules was assayed essentially as described for experiments on the hybrid-tubule-associated protein tau (Butner and Kirschner, 1991). Taxol was sequentially added to 0.1, 1.0, and 10 μM at 2 min intervals to a solution containing 15 μg/ml tubulin, 0.15 mg/ml rhodamine-labeled tubulin in BRB80 (80 mM PIPES [pH 6.8], 1 mM MgCl₂, 1 mM EGTA) with 1 mM GTP. The polymerization reactions were incubated at 37°C for 30 min. The average length of microtubules under these conditions was 9.6 ± 4.5 μm (n = 150), determined by imaging the fluorescent microtubules with a cooled CCD camera and by measuring the lengths with Image 1 software (Universal Imaging, Incorporated). Taxol microtubules were diluted with BRB80, 1 mM GTP, and
from mitotic centrosomes is modulated by a phosphorylated epitope.
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